Elucidating the electron flow in microbial electrochemical technology for broadening the application

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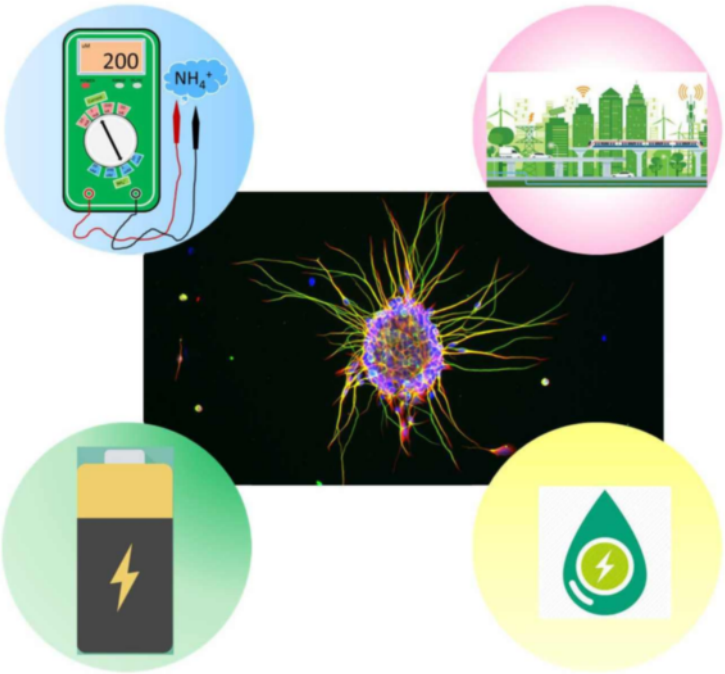

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PhD Thesis
January 2019

DTU Environment
Department of Environmental Engineering

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Preface

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-IV**.

I Zhao, N.N., Li, X.H., Jin, X.D., Angelidaki, I. and Zhang, Y.F. (2017). Integrated electrochemical-biological process as an alternative mean for ammonia monitoring during anaerobic digestion of organic wastes. Chemosphere 195, 735-741.

II Zhao, N.N., Angelidaki, I. and Zhang, Y.F. (2018). Current as an indicator of ammonia concentration during wastewater treatment in an integrated microbial electrolysis cell-nitrification system. Electrochimica Acta 281, 266-273.

III Zhao, N.N., Treu L., Angelidaki, I. and Zhang, Y.F. (2018). Exoelectrogenic anaerobic granular sludge for simultaneous electricity generation and wastewater treatment. (*Under review of* Environmental Science & Technology)

IV Zhao, N.N., Schröder, U., Wichmann, H., Zhang, M.X., Laura, b., Angelidaki, I. and Zhang, Y.F. (2018). Turning methanogenic granular sludge into exoelectrogenic for bio-capacitor application. (*Manuscript*)

In addition, the following publications, not included in this thesis, were also conducted during this PhD study:

Zhao, N.N., Jiang Y.N, Alvarad, M., Treu L., Angelidaki, I. and Zhang, Y.F (2018). Electricity generation and microbial communities in microbial fuel cell powered by macroalgal biomass. Bioelectrochemistry 123, 145-149.

Jin, X.D., Li, X.H., **Zhao, N.N.**, Angelidaki, I. and Zhang, Y.F. (2017). Microbial electrolytic capture, separation and regeneration of CO_2 for biogas upgrading. Environmental Science & Technology 51, 9371-9378.

Jin, X.D., Li, X.H., **Zhao, N.N.**, Angelidaki, I. and Zhang, Y.F. (2017). Bio-electrolytic sensor for rapid monitoring of volatile fatty acids in anaerobic digestion process. Water Research 111, 74-80.

In this online version of the thesis, paper **I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

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This PhD thesis is dedicated to everyone who was in my life in the last three years.

Summary

Microbial electrochemical technologies (MET) have received increasing attention due to the unique merits as environmentally friendly, carbon-neutral technologies for sustainable water treatment, resources recovery, environmental monitoring, and for production of biochemicals and biofuels. In MET, electroactive bacteria (i.e., exoelectrogens), which are able to conduct extracellular electron transfer, are the key engine driving the various applications. Thus, formation of dense, thick and electroactive biofilm on the electrode, is a determining factor for efficient MET processes. However, fundamental understanding on the electron transfer and electroactive biofilm formation is still missing, hindering dedicated development for progressing the state of MET applications in the years to come. The main objective of this thesis is to provide fundamental understanding of the electron flow driving the MET and simultaneously develop novel solutions for key MET applications.

Firstly, efforts were made to develop a microbial electrolysis cell (MEC)-based ammonia sensor to broaden the MET application. An innovative electrochemical cell (EC)-nitrification system was developed for ammonia monitoring. The biosensor was composed of two stages: nitrification stage of ammonia oxidation to nitrate, and cathodic nitrate reduction in EC. Good linear relationship between ammonia levels (0 to 7.1 mM NH_4^+-N) and current signal was consistently obtained independent of the applied voltage and wastewater pH. Subsequently, the biosensor was improved to a novel MEC based sensor which had reduced energy consumption during biosensor operation. Likewise, linear relationship was established between current and ammonia levels (0 and 62.1 mg NH_4^+-N /L). There was no significant difference between the results obtained from biosensor and fast testing kits, indicating the reliable results. However, the anodic biocatalysts were found to be the key limitation to hinder its commercial application. Therefore, much attention has been paid to fundamental areas particularly the anodic biocatalyst.

To obtain an efficient biocatalyst in MET, anaerobic granular sludge (AGS) was selected as potential biocatalyst due to the innate massive microbe concentrations and large surface area. Different strategies were investigated to transform methanogenic AGS into exoelectrogenic. Controlling the anodic potential at +20 mV was found as the optimal strategy to meet the goal of carbon removal along with electron generation. The analysis of microbial

community dynamics proved that electroactive *Desulfurmonadales* spp. increased significantly after the potential control, whereas *Methanosaeta concilii* and *Mesotoga infera* decreased. This result was in good accordance with the increased electricity generation.

Furthermore, the electron storage in exoelectrogenic AGS was studied to elucidate the biological way of electron generation, transportation and storage fundamentally. The results showed that the AGS-based system had a good capacity of electron storage. The optimized capacitance was obtained with 5 min charging and 10 min discharging cycle at +0.2 V. The formal potential observed from cyclic voltammetry analysis was associated to *Geobacter* bacteria. Peaks of cytochromes were also detected by Raman spectra. All these together proved that there was an effective electron flow from the granule to the conductive material (i.e., electrode). Thereof, a potential mechanism of electron storage in such system was disclosed: one contributor is double layer effect and the other one is the cytochromes in exoelectrogens.

Overall, this study broadens the application niches of the technology and also provides novel solutions to the key challenges in MET (i.e. maintaining sufficient amount of biocatalysts). The conducted research work not only provides a capacious application view for MET technology in environmental field, but also brings a fundamental understanding of an effective electron flow in MET.

Dansk sammenfatning

Mikrobielle elektrokemiske teknologier (MET) har fået øget opmærksomhed på grund af de unikke fordele, så som miljøvenlige, kulstofneutrale teknologier til bæredygtig vandbehandling, ressourceudnyttelse, miljøovervågning og bioraffinering af kemikalier og brændstoffer. I MET er elektroaktive bakterier (også kaldet exoelektrogener), som er i stand til at udføre ekstracellulær elektronoverførsel, den helt centrale "motor", der driver de forskellige applikationer. Dermed er dannelse af en tæt, tyk og elektroaktiv biofilm på elektroden en afgørende faktor for effektive MET-processer. Den manglende grundlæggende forståelse af elektronoverførslen og den elektroaktive biofilmdannelse er en væsentlig begrænsende faktor for en dedikeret udvikling af MET applikationer i de kommende år. Hovedformålet med denne afhandling er at tilvejebringe grundlæggende forståelse og elektronstrømmen, der driver MET, og samtidig udvikle nye løsninger til de centrale MET applikationer.

Som det allerførste blev der arbejdet på at udvide MET applikationen ved at udvikle en ammoniak-sensor baseret på en mikrobiel elektrolysecelle (MEC). Den innovative ammoniak-biosensor blev sammensat af to trin: 1) en nitrifikationsfase bestående af ammoniakoxidation til nitrat og 2) en katodenitratreduktion i en elektrokemisk celle (EC). Et godt lineært forhold mellem ammoniakniveauerne (0 til 7.1 mM NH_4^+ -N) og det elektriske signal blev altid opnået uafhængigt af den anvendte spænding og spildevandets pH. Derefter blev biosensoren forbedret til en ny MEC-baseret sensor, som havde et reduceret energiforbrug under biosensoroperationen. Ligeledes blev det lineære forhold etableret mellem det nuværende og ammoniakniveauer (0 og 62.1 mg NH_4^+ -N / L). Der var ingen signifikant forskel mellem resultaterne opnået fra biosensor og fra test kits, som benyttedes som reference-resultater. De anodiske biokatalysatorer viste sig imidlertid at være nøglebegrænsningen for at hindre en kommerciel anvendelse. Derfor er der blevet lagt stor vægt på de grundlæggende områder, især den anodiske biokatalysator.

For at opnå en effektiv biokatalysator i MET, blev anaerob granulært slam (AGS) valgt som potentiel biokatalysator i kraft af den iboende høje mikrobielle koncentration og det store overfladeareal. Forskellige strategier blev undersøgt for at transformere metandannende AGS til en exoelektrogen. Fastlæggelse af det anodiske potentiale ved +20 mV blev fundet som en optimal strategi, for at opfylde målet om fjernelse af kulstof samtidig med dannelse af elektroner. Analysen af dynamikken i det mikrobielle consortium viste, at den

elektroaktive *Desulfurmonadales* spp. steg signifikant efter fastlæggelse af det anodiske potentiale, mens *Methanosaeta concilii* og *Mesotoga infera* faldt. Dette resultat var i god overensstemmelse med den øgede elproduktion.

Derudover blev elektronoplagringen i exoelektrogent AGS undersøgt for at belyse de fundamentale biologiske processer for elektrongenerering, -transport og -opbevaring. Resultaterne viste, at det AGS-baserede system havde en god kapacitet for elektronopbevaring. Den optimerede kapacitans blev opnået med 5 min opladning og 10 min afladningscyklus ved +0.2 V. Det formelle potentiale, der blev observeret fra cyklisk voltammetrisk analyse, var koblet til stilstedeværende *Geobacter*-bakterier. Toppe af cytokromer blev også påvist ved hjælp af Raman spektra. Dette viste, at der var en effektiv elektronstrøm fra granulatet til det ledende materiale (dvs. elektroden). Observationerne afslørede en potentiel mekanisme for elektronopbevaring i et sådant system hidrørende dels fra enr dobbeltlagseffekt, dels fra cytokromerne i exoelektrogener.

Samlet set udvider resultatet af dette forskningsarbejde anvendelsesområderne forteknologien og tilvejebringer også nye løsninger på de vigtigste udfordringer i MET (så som opretholdelse af en tilstrækkelig mængde af biokatalysatorer). Det udførte forskningsarbejde giver ikke blot et omfattende indblik i mulige anvendelsesområder for MET-teknologi på miljøområdet, men giver også en grundlæggende forståelse for hvordan der opnåe en effektiv elektronstrøm i mikrobielle elektrokemiske teknologier.

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Abbreviations

MET	Microbial electrochemical technology
AD	Anaerobic digestion
MFC	Microbial fuel cell
MEC	Microbial electrolysis cell
MES	Microbial electrosynthesis
COD	Chemical oxygen demand
CE	Coulombic efficiency
EET	Extracellular electron transfer
AGS	Anaerobic granular sludge
UASB	Upflow anaerobic sludge blanket
EPS	Extracellular polymer substances
CV	Cyclic voltammetry
EC	Electrochemical cell

1 Introduction

1.1 Background

A prosperous trend of energy consumption over the whole world has appeared in the recent decades. Energy source is generally classified into fossil fuels and renewable sources. The increasing depletion of fossil fuels has accelerated and triggered a global concern due to the limited storage and greenhouse gas release. The exploration of different renewable sources is becoming more meaningful, important and urgent (Steinke et al. 2013). Sun, wind and biomass, which are available over the world, are popular renewable sources (Battaglini et al. 2009). However, sun and wind, as energy sources, have the issues of fluctuation due to the season and day/night cycle variations (Pickard et al. 2009). The unstable energy supply makes it difficult to match the market demand. Comparatively, biomass, with high availability and storage possibility, has its own advantage (Berndes et al. 2003). The biomass, especially the organic waste, is being considered a potential candidate with high values. The organic waste has several attractive features including the ubiquitous, the high chemical energy contained and so on. The use of organic waste is environmentally friendly and considered as a renewable energy source. According to the calculation (Rabaey and Verstraete 2005), 1 kg carbohydrate equals to 1.06 kg chemical oxygen demand (COD), which can be converted to an equivalent power of 4.41 kWh or 1.3×10^7 coulombs (C). Thus, efforts are being made to develop sustainable technologies that can extract huge potential energy from organic wastes.

Microbial electrochemical technology (MET) has emerged as a promising and sustainable technology to treat organics in the recent decades. It mainly utilizes a special group of bacteria to degrade the organics and generate electrons simultaneously. Through MET, the chemical energy stored in organics is converted to various forms of energy including electricity, hydrogen and other valuable bioproducts. MET is a powerful platform to realize the goal of green economic by treating wastes and producing required energy.

1.2 Microbial electrochemistry

1.2.1 Fundamental knowledge

In the nature, various groups of bacteria have been demonstrated to have the ability to produce or transport the electrons via different structures to assist the oxidation-reduction reactions. For example, in the sediment, it has been stated that, there’re groups of cable bacteria to complete the sulfide oxidation

process by transporting the electrons from sulfide to oxygen over centimeters (Bjerg et al. 2018, Jiang et al. 2018). The long distance electron transfer in the cable bacteria is defining the emerging attention to the natural electrochemical communication within cells, which may provide massive information to help us to understand the evolution of earth science. In addition, another outstanding group of microbes which undergo either direct electron transfer or diffusive electron transfer is termed as exoelectrogens (Holmes et al. 2004a, Holmes et al. 2004b, Logan and Regan 2006, Schroder et al. 2015). The metabolism in anode chamber particularly the extracellular electron transfer would be stated later. MET is a platform which mainly exploits the electrochemical communication within exoelectrogens to accomplish bioelectrochemical process (Lovley 2008, Lovley et al. 2011).

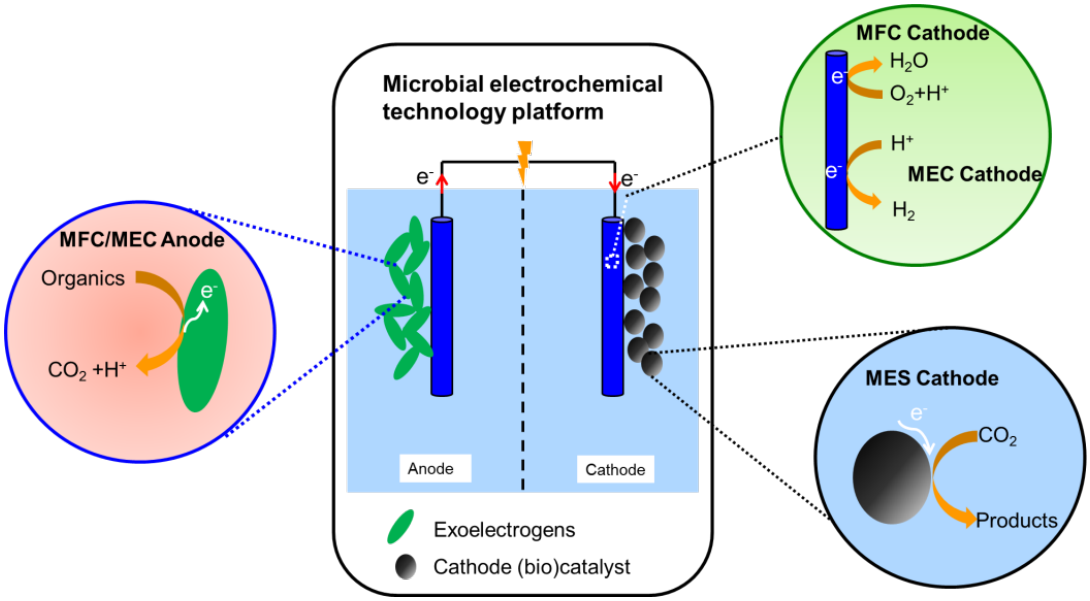

Figure 1. Schematic diagram of different applications of microbial electrochemical systems. MFC: microbial fuel cell; MEC: microbial electrolysis cell; MES: microbial electrosynthesis.

MET utilizes the exoelectrogenic microorganisms to mediate the conversion of chemical energy stored in organic waste into electrical energy and valuable products. Typical MET is usually composed of an anaerobic anode chamber and an aerobic cathode chamber, which are separated by a proton membrane. In the anode chamber, specific microorganisms (i.e. exoelectrogens) anaerobically degrade organic matters into CO_2, protons and electrons. The electrons are transferred to the cathode through external circuit and arrive at cathode to complete the reductive reaction. Through this reaction, the electrons are captured and formed as different forms of energy including electricity, hydrogen, volatile fatty acids and so on. For different purposes, MET

is designed into varied configurations such as microbial fuel cell (MFC), microbial electrolysis cell (MEC), microbial synthesis (MES) and so on (Fig.1). Here we take MFC as an example to explain the working principle of bioconversion process. As shown in Fig.1:

At the anode, exoelectrogens metabolically oxidize the organics to produce protons, CO_2 and electrons, which is further transferred to the anode via redox cofactors such as c-type cytochromes (Hartshorne et al. 2007) or special biological structure (nanowires) (Lovley and Malvankar 2015) or mediators (Schmitz and Rosenbaum 2018). Driven by an external circuit (load), the electrons arrive at the cathode, where an oxidant (normally oxygen) is reduced with electrons and protons exchanged from anode via membrane.

The reactions are displayed as below:

Anode: $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$

Cathode: $6O_2 + 24e^- + 24H^+ \rightarrow 12H_2O$

Total: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + ElectricalEnergy \qquad \Delta G^0 \approx -2840kJ/mol$

Different from MFC, MEC mainly focuses on the hydrogen production with electrochemically assistance (Logan et al. 2008). In an MEC anode, the same exoelectrogens transfer the electrons produced during substrate oxidation to the anode, and thereafter the electrons are flowed to the cathode driven by external electrical force, where protons combine with electrons to form hydrogen. An external power source (0.2-0.8 V) is necessary to provide extra energy for driving the hydrogen production reactions at cathode (Zhang and Angelidaki 2014). The value of added power is relatively lower compared to typical water electrolysis (>2.1 V). In a similar context, MES emerged as a platform to biocatalyze CO_2 into fuels or other value-added chemicals at low potentials via chemolithoautotrophic bacteria (Katuri et al. 2018).

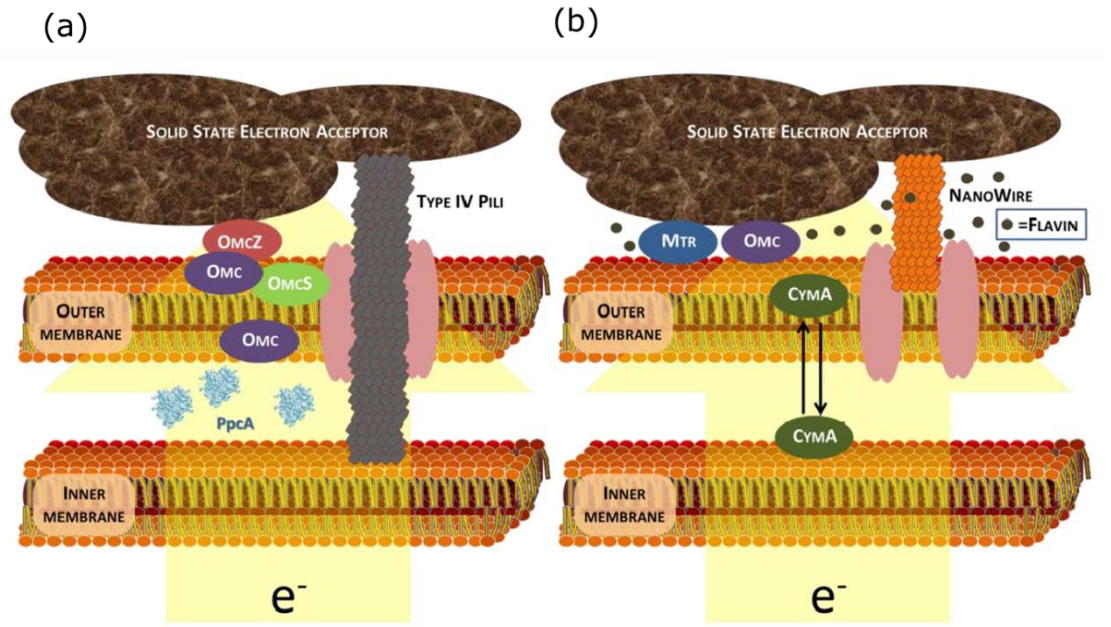

Figure 2. Schemes of EET in *Geobacter* (a) and *Shewanella* (b). (Kumar et al. 2017).

In spite of the configurations, they all share one common principle in the anode chamber. Regarding the extracellular electron transfer (EET) process within cells or from cell to solid electron acceptor, there are three possible ways of exoelectrogens used for transporting electrons to the cells nearby or to the electrode, including (1) through outer membrane-bound redox protein c type cytochromes and other redox proteins (i.e., OmpB and OmpC); (2) through conductive structures (nanowires); (3) via exogenous mediators or self-excreted small molecules as the electron shuttle (Kumar et al. 2017, Lovley et al. 1996, Newman and Kolter 2000, Shi et al. 2007, Tao et al. 2015). To date, the most two popular prototype strains in EET research are *Geobacter* and *Shewanella*. The EET of both strains is displayed in Fig.2 (Kumar et al. 2017). They both utilize c type cytochromes to transport electron to electron acceptors. C type cytochromes refer to the proteins which contain multi-heme. *Geobacter sulfurreducens* possesses 111 genes encoding c type cytochromes. Among them, 73 contain over two heme groups. In particular, one contains as many as 27 heme groups. Similarly, *Shewanella oneidensis* has 39 genes encoding c type cytochromes, and 14 of them contain over four hemes. The detailed structures of these cytochromes have been reviewed previously (Mowat and Chapman 2005). The key cytochromes of both bacteria in performing EET have been identified and investigated through the regulation of gene expression. In *Geobacter*, a variety of outer membrane-bound cytochromes (OMCs), including OmcB, OmcE, OmcS and OmcZ contribute to the outer membrane EET. OmcS has been stated that it is

involved in the direct electron transfer from outer membrane to the electrode, and meanwhile they have the ability to facilitate the long-range electron transfer via the type IV pili (Holmes et al. 2006). OmcB contributes to the intermediary electron transport from periplasm to other OMCs (Mehta et al. 2005). OmcZ is crucial to outer membrane EET (Malvankar et al. 2011). However, the function of OmcE in EET is still in debate so far (Strycharz-Glaven et al. 2011b). Genetic engineering approaches have been also applied to study the roles of cytochromes in *Shewanella* EET. It was concluded that series of protein-protein interactions accomplished the EET of *Shewanella*. First, the electrons generated from bacteria metabolism were transported to the terminal reductases in periplasm via CymA. This step is considered indispensable in *Shewanella* EET because a genetic deletion of CymA resulted in around 80% decreasing in current (Yang et al. 2012). Second, the electrons were passed from the reductases to the outer membrane proteins such as MTRs and OMCs. Finally, these outer membrane proteins transfer the electrons to the electrode or electron shuttles to complete the EET process. As deletion of MtrC led to over 90% decrease in current generation, MtrC is noted as one of the most important proteins involved in *Shewanella* EET process (Snider et al. 2012).

1.2.2 Technology applications

Based on the working principle of MET, different applications have been investigated to fit more niches, including electricity generation, organic removal, nutrient recovery, biosensor, heavy metal pollutants removal, water desalination, charge storage and so on (Logan and Rabaey 2012, Wang and Ren 2013). Our group has proposed innovative MET-based systems for various applications such as for electricity generation (Zhang and Angelidaki 2012b), wastewater treatment (Zhang et al. 2011b), biosensor monitoring (Jin et al. 2016), nitrogen recovery (Zhang and Angelidaki 2012a) and AD biogas upgrading (Jin et al. 2017b). In the current thesis applications related to biosensor development, wastewater treatment and biocapacitor will be mainly reviewed.

1.2.2.1 Biosensor

In the recent decades, MET has been widely applied into biosensor applications, either based on the power generation for remote electronic devices (Donovan et al. 2011, Zhang et al. 2011a) or based on the working principle which links the current to oxidant/reductant (Zhang and Angelidaki 2012c, Zhang and Angelidaki 2011). Here we mainly display the latter one.

Based on the basic working principle, MET has been designed into different configurations to realize the components monitoring. In all configurations, they share one common principle in anode: the oxidation of organics by the microorganisms to generate the electrons. According to the intrinsic reaction, the amounts of electrons released from organic oxidation should be proportional to the organic loading. Several works have been performed to demonstrate its feasibility to be employed as volatile fatty acids monitoring (Kaur et al. 2014, Kaur et al. 2013), and several modifications have been made to reduce the cost and optimize its monitoring accuracy regarding to the configurations and monitoring range (Jin et al. 2016, Jin et al. 2017a). In addition to exploiting the anodic reaction to design a biosensor, the cathodic reaction has also been considered to fulfil its feasibilities to work as a monitor. The electrons flowing from the anode lead to the cathodic oxygen reduction, which links the current to oxygen levels. It enables the MFC employ as a biosensor for dissolved oxygen (Zhang and Angelidaki 2012c). Moreover, due to the intrinsic features, MET has been also developed for monitoring pH and temperature (Rabaey et al. 2004), microbial activity (Zhang and Angelidaki 2011), toxicity of heavy metals (Jiang et al. 2015).

Compared to traditional off line sensors or other enzyme-based biosensors, the MET-based biosensors have several advantages including cost-effectiveness, higher stability and sensitivity, and environmentally sustainability (Ivars-Barcelo et al. 2018). Considering the superior advantages and performance of MET-based biosensor, online monitoring of ammonia, which has never been investigated, is developed in this project.

1.2.2.2 Wastewater treatment

Due to the increasing environmental resource scarcity and electricity demand, the wastewater is recognized as one of the energy-rich resources (Logan and Rabaey 2012). As the attractive features of extracting energy from the wastewater in MFC, extensive work has been conducted to investigate and optimize the wastewater treatment in MFC (He et al. 2017). Conventional way to treat wastewater is aerobic activated sludge process, which is characterized as energy intensity, large residuals generation and limited nutrient recovery. It's pointed out that in US, running a typical domestic wastewater treatment plant required 0.5 kWh/m^3, and wastewater treatment only consumed nearly 3% of the total electricity consumption (McCarty et al. 2011). In this context, MFC, as an integration of electrical energy generation and organic removal, is quite promising. Compared to conventional biological

way of wastewater treatment, MFC has three outstanding features including energy saving, less sludge production and energy production.

Table 1. Summary of all types MFCs for wastewater treatment

Types of MET	Substrate	Max. Power/Current density	COD removal efficiency	Coulombic efficiency	Ref.
Artificial wastewater					
Single chamber MFC	acetate	0.8 A/m^2	91	40	(Zhang et al. 2015)
Dual chamber MFC	glucose	216 W/m^3	41	15	(Rabaey et al. 2003)
U-type MFC	sucrose	25 W/m^3	90	51	(He et al. 2006)
Cassette-electrode MFC	Yeast, starch	150 mW/m^2	80	20	(Miyahara et al. 2013)
Domestic wastewater					
Photosynthetic MFC		400 mW/m^3	87	-	(Angioni et al. 2018)
Up flow membraneless MFC		268.5 mW/m^2	67	53.8	(Jiang 2017)
Industrial wastewater					
Single chamber MFC	dairy	5.7 W/m^3	80	75	(Ayyaru and Dharmalingam 2011)

H-type MFC	agriculture	37 mW/m^2	73.7	37	(Fernando et al. 2012)

So far, in the field of wastewater treatment, different configurations of MFC have been explored and applied in various types of wastewater (Wang and Ren 2013). Here, we displayed a table summarizing the application of MFC in purifying wastewater (Table 1). The performance was evaluated in terms of chemical oxygen demand (COD) removal, coulombic efficiency (CE) and power/current density. There are several parameters usually used to determine the performance of wastewater treatment, such as COD/pollutant removal rate, CE and power/current density. It's often traded off between COD removal and electricity generation. It's always expected a good COD removal coupled with high electricity generation (a high CE). High CE indicates a good bioconversion of organic to current. However, in the scaling up, when volume is increased, the internal resistance is elevated (i.e., low electron transfer in anode), leading to a low CE (Logan 2010). That's one of the issues hinder the real upscaling from several litters to hundred litters of wastewater. On one hand, accelerating electron transfer in anode has been addressed as one of the most important factors to enhance the COD removal and electricity generation. Cytochromes have been demonstrated widely to be capable of transporting electrons from cells inside to outside and further electrode contact (Lovley 2017, Ueki et al. 2018). The c-type cytochromes allow an effective electron transfer in biofilm matrix, and thereby greatly improve the carbon degradation. Not only c-type cytochromes, but also other special biological structure (i.e. nanowire) has been demonstrated as another important conductive material between cells and electrodes or within biofilm (Lovley and Malvankar 2015, Malvankar and Lovley 2014). On the other hand, the biomass content determines how much microbes are involved in the biological process. The more, the better. Thus, forming a thick and dense electroactive biofilm is of high importance to achieve an efficient biological system.

1.2.2.3 Biocapacitor

It has been demonstrated that the c-type cytochromes that not only transfer the electrons, but also have the capability of electron storage temporarily (Liu et al. 2011, Malvankar et al. 2011, Ren et al. 2015, Strycharz-Glaven et al. 2011a). The nanowire in/on the membranes and phosphor-lipid bilayer structure of exoelectrogens enable the exoelectrogenic microbe work as a biocapacitor (Strycharz-Glaven et al. 2011a). Based on these solid research works, an electron charge accumulation was separated as a branch to expand the application of MET. Particularly, firstly, MET extracts electrical energy

from wastewater; secondly, the extracted energy could be stored in the exoelectrogens for a while to provide power when it's needed. In this way, the energy is produced and preserved.

To optimize its performance of electron storage and commercialize its application as biocapacitor, some of researches pay attention to modify the electrodes with exoelectrogens, taking full advantage of cytochromes. In 2012, Malvankar et al. (Malvankar et al. 2012) firstly developed a supercapacitor assembled based on *Geobacter sulfurreducens* biofilms, and demonstrated the faradic reactions of the cytochromes exhibited large pseudocapacitance compared to a synthetic supercapacitor. This is the first study to measure the in vivo capacitance of *G. sulfurreducens*. Meanwhile, another group (Bonanni et al. 2012) investigated the charge accumulation in *G. sulfurreducens* and differentiated the stored charge inside the cells and outside the cells in the conductive biofilm matrix by fitting a model. Subsequently, Buisman's group developed another integrated electron storage system which contained a capacitive electrode colonized with electrochemically active biofilm (Deeke et al. 2012). In their research, they conducted cycling test (charge-discharge) on the capacitive bioanode and non-capacitive bioanode, and demonstrated the capacitive bioanode outperformed non-capacitive bioanode through all cycling tests. Later on, in 2013, same group explored the optimal thickness of capacitive layer on the charge storage of the bioanode in MFC (Deeke et al. 2013). In 2015, activate carbon granules were firstly demonstrated as fluidized capacitive bioanode colonized by electroactive microorganisms in an MFC (Deeke et al. 2015). Following the concept of fluidized capacitive bioanode, further work was performed to investigate the capacitive performance of single electrochemically active carbon granule, and results illustrated a better performance of charge accumulation in activated carbon granule than that in graphite granule (Borsje et al. 2016). Similar capacitive properties were also obtained in a previous study which explored the electron storage in the activated carbon granules colonized with exoelectrogens (Liu et al. 2014). In addition to the *G. sulfurreducens, Shewanella oneidensis* has also been proved to have the ability to store charge (Uria et al. 2011).

The aforementioned work enables MET not only generate the electricity from wastewater, but also preserve the energy either in granular bioanode or in pure electrochemically active species. It suggests a good development of MET to occupy more application niches in material/environment/energy science.

1.2.3 Challenges of microbial electrochemical technology (MET) platform

Before a successful commercial application of MET technology, some barriers of the technology should be taken into account. These barriers include the high operational cost and low power output. Compared to the conventional activated sludge treatment, it normally costs over 30 times higher in MET system. The capital cost is principally derived from the configuration and treatment capacity. Generally, the high cost in MET is caused by the expensive electrode materials, such as the catalyst. On the one hand, one principle to choose an ideal electrode is to make sure a good conductivity between the electrode and the microorganisms. On the other hand, a good electrochemically active biofilm in the anode would benefit a higher power/voltage output. Taking together, developing a thick, dense and electroactive biofilm has become a determining factor for an efficient MET process, and further facilitate the commercialization of this technology.

So far, there are various approaches applied to form an ideal biofilm, including 3D electrodes (i.e., 3D porous carbon) for offering a large surface area for bacteria growth (Bian et al. 2018), manipulating anodic potential for supporting energy for bacteria uptake (Schroder 2007). On the one hand, amounts of work have been performed to investigate the performance of 3D electrode materials, which provided a large surface area for bacterial growth and biofilm attachment. Thick biofilm means more exoelectrogens would be involved in the exoelectrogenic process (the metabolism), which is closely associated with the organic degradation. On the other hand, when elevating the anode potential, more energy would be utilized by the bacterial to maintain the growth and thus facilitate the propagation. In addition to forming a thick biofilm, accelerating electron transfer within the cells/biofilms and from cells to electrode would be also effective. It was found that the EET-proteins increased with increasing anodic potential (Carmona-Martinez et al. 2013).

1.3 Anaerobic granular sludge (AGS)-based anaerobic digestion

Anaerobic digestion, which mainly utilizes various groups of microbes to convert organics to biomethane for further energy use, is another popular method to treat wastewater. Compare to the aerobic wastewater treatment, it has the advantages of less sludge production and low energy consumption. Upflow anaerobic sludge blanket (UASB) reactor, as an extensively used type of anaerobic digestion system, was developed in the late 1970s (Lettinga et

al. 1980). The anaerobic granular sludge (AGS) is formed in the UASB reactor. The granulation process is quite complex and affected by many physicochemical factors. The inorganic composition of AGS contains calcium, potassium and iron, which are termed as the ash content. The organic contents of AGS are extracellular polymer substances (EPS), which play a very important role in forming and maintaining the granule structure. EPS mainly contain polysaccharides, proteins, lipids, phenols and nucleic acids along with small amounts of organic debris and lysed cells. It has been shown that EPS are able to protect bacteria from the surroundings and the interactions with granules benefit the sludge granulation (Forster 1991, Morgan et al. 1991). Regarding the granular structure, cavities and holes are usually observed on the granule surface via scanning or transmission electron microscopy. These cavities or holes are used for transporting the substrate in and out (Macleod et al. 1990, Morgan et al. 1991).

With such granular structure, the AGS-based reactor has several advantages in addition to the aforementioned good merits of anaerobic digestion system, mainly including 1) an excellent performance of long solid retention time even at high organic loadings; 2) low hydraulic retention time at high-rate; 3) improved sludge dewaterability. However, it should be remarked that this system still encounters several challenges including 1) long start-up period; 2) low performance at low organic loadings; 3) post treatment for water disposal; 4) low pathogen and nutrient removal.

Thus, innovative technologies, which can take advantages of AGS and in big scene bring benefit to environment, are of high importance and urgently needed.

1.4 Objective and thesis structure

1.4.1 Objective

This project aims to expand more application niches for MET including biosensor online monitoring, wastewater treatment and electron storage device, and provide a comprehensive fundamental understanding of electron transfer. In this case, to achieve a good performance in each application field and get a deep insight into the electroactive biofilm formation, optimal strategies were investigated and employed. Based on the main objectives, we not only fully met the three goals of applying MET into biosensor, wastewater treatment and biocapacitor, but also provide a fundamental understanding of electron transfer pathway from biological and electrochemical viewpoint.

Specifically, the detailed objectives are shown below

- Develop an integrated electrochemical cell-nitrification system for online ammonia monitoring, realize a high conversion rate of ammonia to ni-trate in the nitrification stage, establish a good relationship between ammonia levels and current signal, and analyze the system applicability with real AD effluents. (Paper I)
- To reduce the cost and realize an environmentally friendly and sustainable ammonia biosensor, a microbial electrolysis cell-nitrification system was constructed for ammonia online monitoring, in which ammonia levels were linked to the current signals. And study the influencing parameters and verify its accuracy in waste streams. (Paper II)
- Decipher the determining factor for efficient MET process, followed by utilizing AGS as electroactive biocatalyst to target on the key limiting factor. And demonstrate the good performance of wastewater treatment in exoelectro-genic AGS regarding to the high organic removal along with electricity generation, and reveal the microbial community dynamics during the tran-sition of methanogenic to exoelectrogenic AGS. (Paper III).
- Develop a good electrochemical activity of AGS in a new developed bio-capacitor system by exploiting the optimal strategy proposed in Paper III. And establish a good capacitance behavior of exoelectrogenic AGS by charging-discharging test, and optimize the new biocapacitor performance by changing the multi-parameters. (Paper IV)
- Depict the electrochemical and microscopic behavior of single AGS and identify the microbial community change under optimal strategy operation. (Paper IV)
- Propose the working mechanism of electron storage in exoelectrogenic AGS. (Paper IV)

1.4.2 Structure

In Chapter 2, an integrated electrochemical cell-nitrification system is set up and investigated its feasibility for ammonia online monitoring. Ammonia is converted to nitrate via a nitrification process, and then further linked to current signals in the electrochemical cell. The mechanism of the monitoring process was explained and the effect of varied operational conditions on the biosensor performance was investigated. Considering the limitations of the aforementioned ammonia biosensor, a more advanced and environmentally friendly system for ammonia biosensor was exhibited. Good correlations

between ammonia concentrations and current responses in a microbial electrolysis cell-nitrification system are reported. The effect of external power supply and pH on ammonia biosensor performance was studied, and accuracy of the developed biosensor was verified by testing it with real waste streams. Finally, the significance and future perspectives of the ammonia biosensor is discussed. The electroactive biofilm was deciphered as the determining factor for an efficient MET process. Thus, in Chapter 3 and 4, fundamental research of electron flow in anodic biofilm was explored.

In Chapter 3, AGS was selected as the biocatalyst due to the innate massive microbes. Three different strategies are investigated and one is selected as a successful strategy to shift the methanogenic AGS to exoelectrogenic granules. High organic removal and current generation were achieved in the exoelectrogenic AGS. Morphology of AGS was characterized to provide deep insights of its structure and biofilm immobilization. Finally, microbial community composition was compared between methanogenic AGS and exoelectrogenic AGS, and results are provided to understand the key role of microbes in the organic removal and electricity generation of exoelectrogenic AGS.

In Chapter 4, based on the optimal strategy demonstrated in Paper III, exoelectrogenic AGS was successfully developed for electron storage study. The capacitive behaviour of exoelectrogenic AGS was evaluated in cycling test (charge-discharge), and multi-parameters are studied to optimize the electron storage capacity. To better interpret the electron storage process, single AGS was electrochemically characterized in terms of cyclic voltammetry and Raman spectroscopy. Furthermore, microbial community change was analyzed to provide biological information under exoelectrogenic conditions. Finally, based on all the results, a mechanism towards to explaining how the electrons are stored in the biocapacitor was proposed and discussed.

In Chapter 5 and 6, conclusions of the whole project and future perspectives are illustrated, respectively.

2 MET applied as ammonia biosensor

Ammonia is often derived from various processes such as food factory, fertilizer production and AD reactor. Ammonia is often toxic to living cells. It would affect lake water quality, human health and methanogenic activity in biogas reactors. Therefore, the ammonia monitoring is of high importance nowadays.

So far, the most attractive device/approach for ammonia monitoring is ion selective-based ammonia sensor (Zhou and Boyd 2016). This ammonia sensor has been widely commercialized due to its reliability/accuracy. However, there're still some challenges that need to be addressed. For example, this commercialized ammonia sensor is usually used for off-line and in some cases it can't realize real in-situ monitoring. Also the cost including sampling and maintenance is expensive. Recently, MET attracted our attention since it has been widely developed for other compound sensors including volatile fatty acids monitoring (Jin et al. 2016), microbial community monitoring (Zhang and Angelidaki 2011) and dissolved oxygen monitoring (Zhang and Angelidaki 2012c). Particularly, the principle of oxygen sensor was to establish a correlation between current and oxygen according to the reduction reaction of oxygen in cathode. Inspired by that work, considering the need of alternative ammonia sensor, how to make a current signal from ammonia levels became attractive to us. After full consideration, we combined the cathodic reduction with nitrification process to first oxidize ammonia to nitrate, which would be further reduced with electrons. In this case, the current response was linked to ammonia levels. Based on the hypothesis, in this chapter, we conducted two packages of work: first we develop an EC coupled with nitrification system for online ammonia monitoring in AD; secondly, to reduce the cost, we developed a microbial EC coupled with nitrification system-based ammonia biosensor.

2.1 Ammonia biosensor in an integrated electrochemical cell-nitrification system

2.1.1 The importance of ammonia monitoring in AD system

In AD system, the ammonia is produced from the protein degradation, which suppressed the biogas production via microbial toxicity (Abass et al. 1998, Tian et al. 2018). It was reported that even at low concentrations of free ammonia (i.e. 100 mg-N L^{-1}), it was problematic to maintain a good performance of methanogens in unadpated AD reactor (Hansen et al. 1998). Thus,

it’s significantly important to monitor ammonia concentrations in AD in order to maintain a stable and effective operation.

Currently, there’re several ways to test ammonia levels including colorimeter method (Nessler and phenate methods), ion-selective electrode for ammonia and fast testing kits (Krug et al. 1979, Le and Boyd 2012, Zhou and Boyd 2016). However, there’re several drawbacks among these methods: 1) the easily disturbance by the color or turbidity in terms of the accuracy of colorimeter method; 2) the issues of toxic reagents using in the Nessler method; 3) extra sample preparations are needed for most of the colorimeter method; 4) the performance of ion-selective electrode is strongly dependent on the conductivity of samples particularly the concentrations of sodium and potassium. All these issues result in an urgent need to develop an environmentally friendly and cost-effective method to realize on-line ammonia monitoring.

In this context, electrochemical system attracted our attention since it has high selectivity and sensitivity (Ning et al. 2017, Ribeiro et al. 2012, Zhybak et al. 2016). The common electrochemical ammonia sensors are based on the ion transfer reactions through various electrolyte interfaces (Abass et al. 1998, Bertocchi et al. 1996, Herzog 2015). Although with so many good merits, it has to be pointed out, that these methods also require tedious and complicated fabrication processes with large consumption of nanomaterials in some cases.

2.1.2 An innovative concept to develop an on-line ammonia biosensor

Base on the successfully reduction of nitrate in the cathode of an EC, a hypothesis was proposed: we could combine a biological nitrification process together with EC cathodic reduction reaction to establish a relationship between current signals and ammonia levels. Specifically as shown in Fig.3, in the first stage, the ammonia in AD samples was converted to nitrate; secondly, the nitrate-rich sample was reduced with the electrons from circuit. In this way, the current value (amounts of electrons) would be associated with ammonia concentrations.

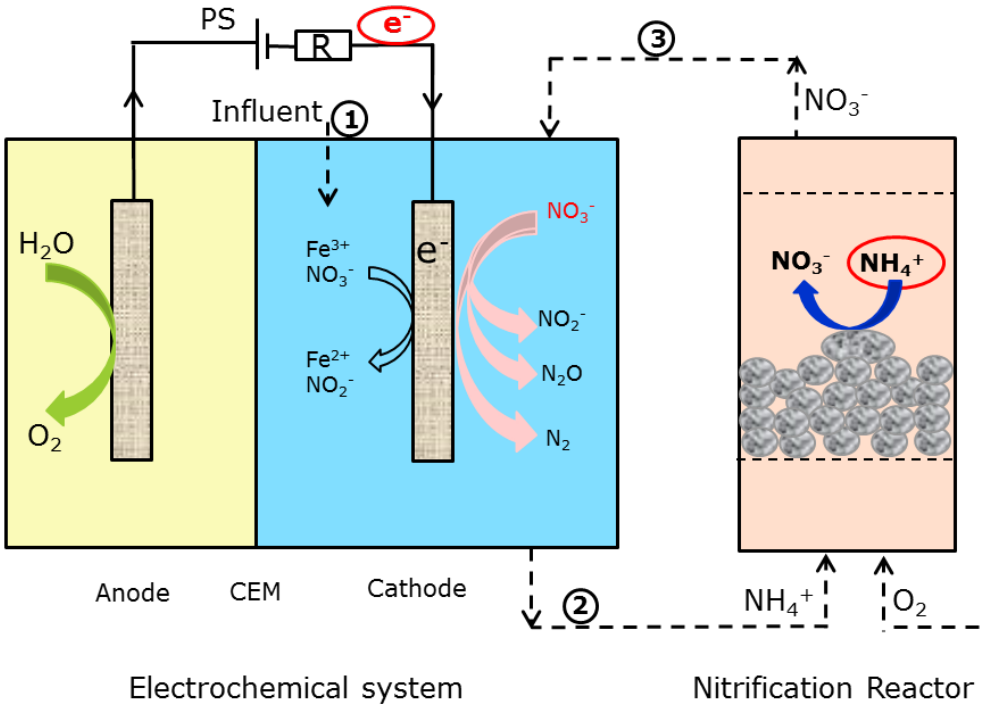

Figure 3. Schematic diagram of the integrated EC-nitrification system. CEM: the cation exchange membrane. (Zhao et al. 2018a)

For the first stage, the correlation between initial ammonia concentrations and produced nitrate levels was shown in Fig.4. The tested ammonia levels ranged from 0 to 7.1 mM NH_4^+-N, which was converted to 0 to 6.9 mM NO_3^--N. A stable conversion rate was obtained, indicated as the linear line. The slope is 0.9534, suggesting a nearly fully oxidation reaction (Tarre and Green 2004). After the first stage, the nitrated-contained waste was introduced to the cathode of EC (purging with N_2 to remove oxygen). It was observed a fast current response in the EC circuit to a stepwise increase from 0 to 7.1 mM NO_3^--N within five minutes (Fig.4b). Based on the effective conversion rate of ammonia to nitrate and the positive current response to nitrate levels, we established a linear relationship between ammonia and current levels (Fig.4c). The linear relationship demonstrated our hypothesis and confirmed the feasibility of the new developed ammonia biosensor. Compared to other conventional testing kits, obvious advantage was no toxic reagents and comparatively rapid monitoring time. Compared to other EC-based sensor, this new biosensor has widened the monitoring range of ammonia levels, and no complex process was required.

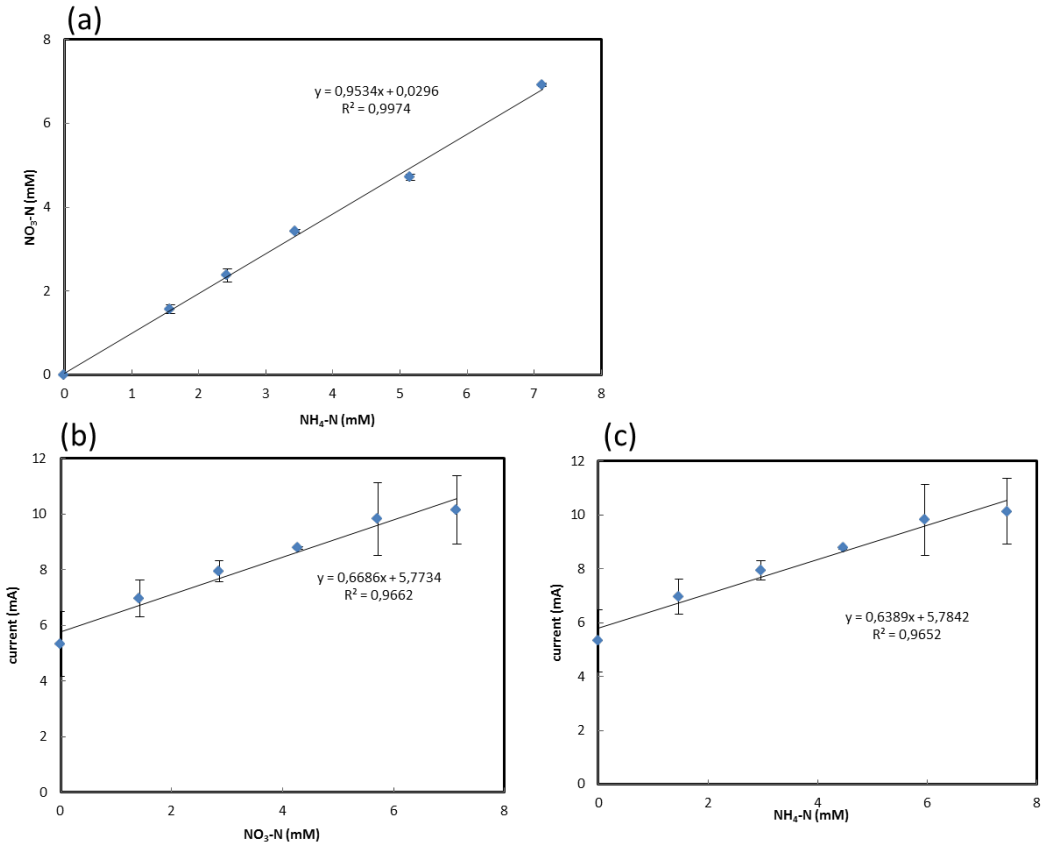

Figure 4. The correlation between initial ammonia level and accumulated nitrate concentration in nitrification reactor (a). The linear relationship between current and nitrate levels (b) and ammonia levels (c) at external power supply of 1.8 V and pH 6. (1.4 to 7mM NH_4^+-N) (Zhao et al. 2018a)

2.1.3 Ammonia biosensor performance at various conditions

To investigate how the performance was affected by the external voltage, wastewater pH and other potential oxidized species, we applied various operational conditions to the ammonia biosensor. As depicted in Fig.5a, in spite of the external voltages, there was always a positive linear slope between current response and ammonia levels. The highest slope was observed when the applied voltage was 2.3 V, indicating a higher current generation at 2.3 V. It was explained by that with higher applied voltage, there would be higher amounts of electrons. Notably, at all applied voltages, the biosensor exhibited high linearity over 90%, demonstrating a good performance of the new biosensor at different voltages. Considering the AD digesters usually have various pH range (Cook et al. 2017), different pH ranging from 4 to 8 was applied to the sensor to investigate the sensor performance (shown in Fig.5b). Similarly, the linear correlation for current signals with ammonia levels was

observed for all pH conditions. A higher slope was likely to appear in neutral and weak alkaline condition.

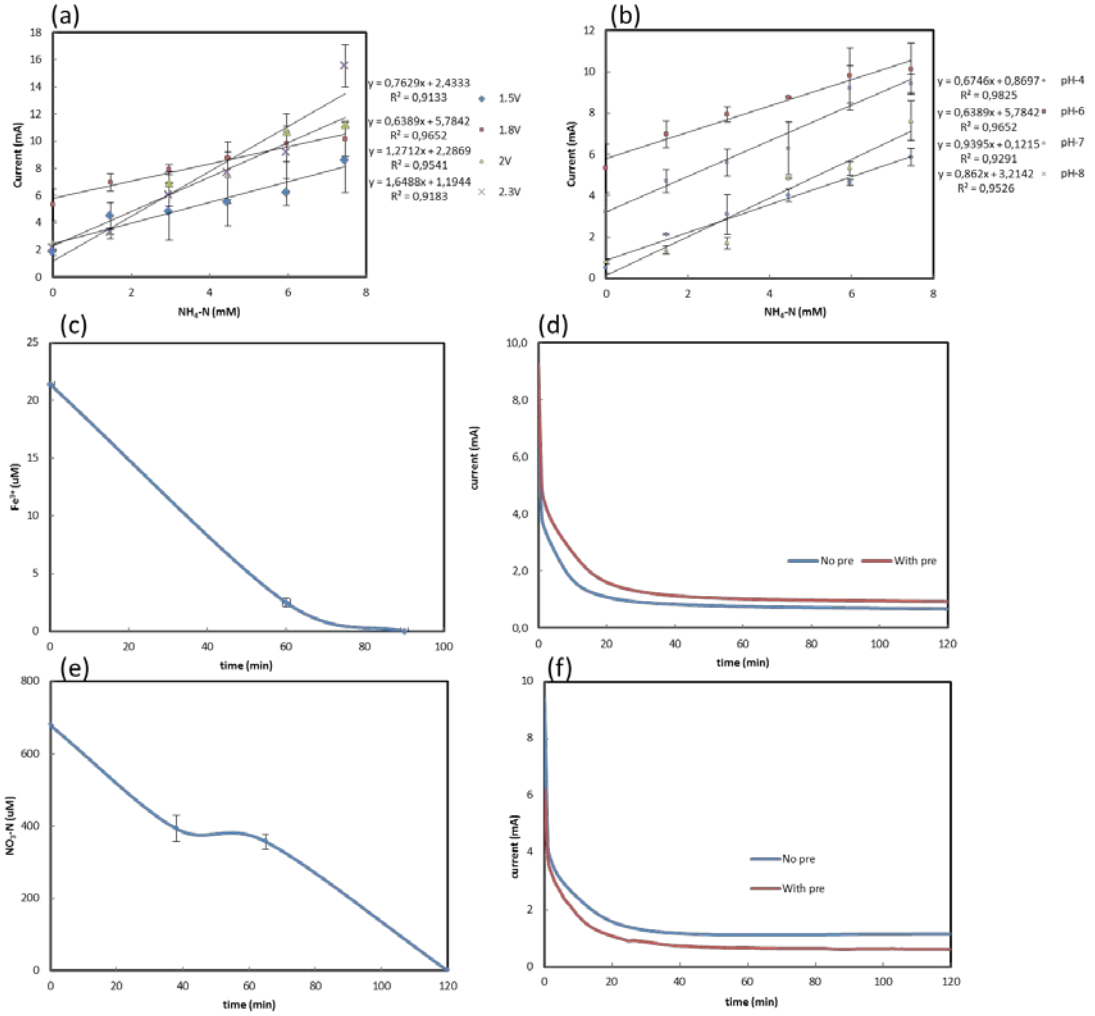

Figure 5. Effect of external applied voltage (a) and pH (b) on the performance of ammonia sensor. The change of Fe^{3+} concentration (c) and current generation (d) over time in EC reactor. The operational parameters: external voltage of pretreatment is 4 V, initial pH is 6 and initial NH_4^+-N of 6 mM in synthetic water. The change of NO_3^--N concentration (e) and current generation (f) over time in EC reactor. The operational parameters: external voltage of pretreatment is 4 V, initial pH is 6 and initial NH_4^+-N of 80 mg/L in synthetic water. (Zhao et al. 2018a)

Since the second step of the sensor working principle was based on the nitrate reduction in EC cathode, it's meaningful to consider the effect of other potential electron acceptors on the biosensor performance. Therefore, here we designed an extra post-treatment of the sample before staring the first stage and second stage to exclude the effect of potential electron acceptors. Fig.5c shows the Fe^{3+} removal in EC cathode and current generation difference between the samples with and without Fe^{3+} removal. From Fig.5c, it's clearly

that the Fe^{3+} was totally removed in 90 minutes via cathodic reduction when an applied voltage of 4 V was applied. To see the different current generation in both conditions, the wastewater with and without pretreatment were separately introduced into EC cathode and current was recorded (Fig.5d). Significant difference at 99% confidence level was found between the current responses in two cases by using student's test. These results proved an effective post-treatment before nitrification to abolish the other electron acceptor disturbance. Likewise, NO_3^- is not negligible since the nitrate ions in the initial samples would result in errors if it's not removed before entering the nitrification stage. Similar nitrate removal and significant difference were obtained (Fig.5e and f). To short conclude, introducing the wastewater into EC cathode before entering the nitrification was effective to eliminate other potential oxidized species effect. Coupled with this post-treatment stage, the stability and sensitivity of this new biosensor was improved. One thing needs to be addressed is that the post treatment may be not necessary for AD process due to the absence of these ions (Fe^{3+} and NO_3^-), but it still offers a new insight into more potential application niches of the biosensor, i.e. domestic wastewater.

2.1.4 Accuracy of biosensor treated with real AD effluent

Table 2. Determination of NH_4^+-N in real waste streams by EC-nitrification based biosensor and ammonia testing kit. (Zhao et al. 2018a)

sample	NH_4^+-N[a]	NH_4^+-N[b]	pH	Conductivity (us/cm)
AD effluent 1	1542.30 ± 5.12	1647.8 ± 13.18	8.87 ± 0.10	1690 ± 2
AD effluent 2	917.24 ± 2.16	987.5 ± 20.04	8.20 ± 0.10	1329 ± 5

a: tested by fast kits. b: measured by sensor.

The applicability of new biosensor was evaluated by the real AD digestate. The results obtained from our sensor and from fast testing kits were compared in Table 2. To investigate the difference between two series of data, anova was performed. There was no significant difference between the results obtained from fast kits and sensors ($P > 0.05$) at 95% confidence level. The results proved that the new developed biosensor has a good accuracy and wide range of ammonia concentrations.

To short conclude this work, it for the first time demonstrated the feasibility of EC-based coupled with nitrification stage for online ammonia monitoring during AD process. The monitoring time was within 5 minutes and ammonia level range was 0 to 7.5 mM NH_4^+-N. The biosensor performance was independent of external applied voltage and pH. Moreover, the interface of other potential electron acceptors was eliminated by the post treatment of EC. In

the end, the sensor exhibited reliable results and high sensitivity to real AD digesters. Though promising, the operational cost especially the external applied voltage should be taken into account, and more advanced approaches should be developed to reduce this section cost.

2.2 Improving ammonia biosensor in a microbial electrolysis cell-nitrification system

2.2.1 A new developed concept describing ammonia detection in a microbial electrolysis cell-nitrification system

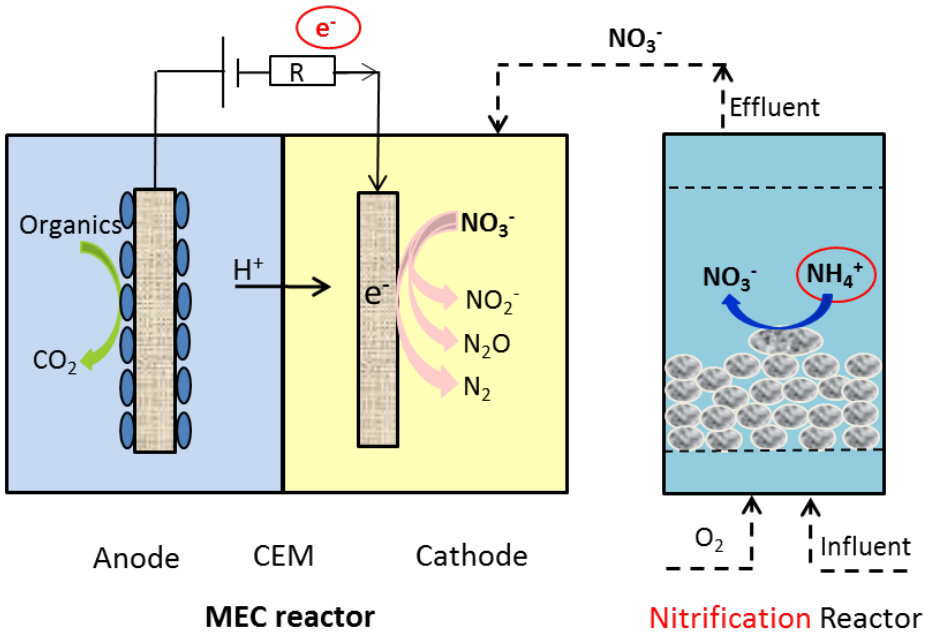

Figure 6. Schematic diagram of the integrated MEC-nitrification system. (Zhao et al. 2018b)

In Section 2.1, we described an EC-nitrification ammonia biosensor. It exhibited high accuracy but the cost of external applied voltage still needed to be addressed. To offset this cost, an advanced system which includes a microbial electrolysis cell-nitrification configuration was developed for online ammonia monitoring (Fig.6). The hypothesis was still based on the nitrification conversion of ammonia to nitrate together with cathodic nitrate reduction. Differently, in microbial electrolysis, part of the energy provided by the external voltage was compensated by the energy from wastewater. In MEC anode, the chemical energy stored in the wastewater was converted to electrical energy via exoelectrogenic metabolism. This part of the extracted electrical energy could compensate parts of energy consumption for desirable products such as hydrogen (Zhang and Angelidaki 2016). Inspired by the working

principle of MEC, it was motivated to design a MEC-nitrification ammonia biosensor in this section.

2.2.2 Biosensor performance by linking ammonia concentration to current level

The nitrification process was evaluated in terms of the conversion efficiency. As demonstrated in Fig.7a, a good conversion efficiency of ammonia to nitrate was achieved. After the first stage, nitrate-rich wastewater was introduced to MEC cathode. From Fig.7b, we could see the increasing current response with elevating nitrate levels. For each level, the current tended to reach a plateau after 15 minutes. We got the current data after 30 minutes operation and established a good linear relationship between current signals and nitrate concentrations. Similarly, the relationship between current and ammonia was established in Fig.7c. The detection range of ammonia in this biosensor was 0 and 62.1 mg NH_4^+-N /L.

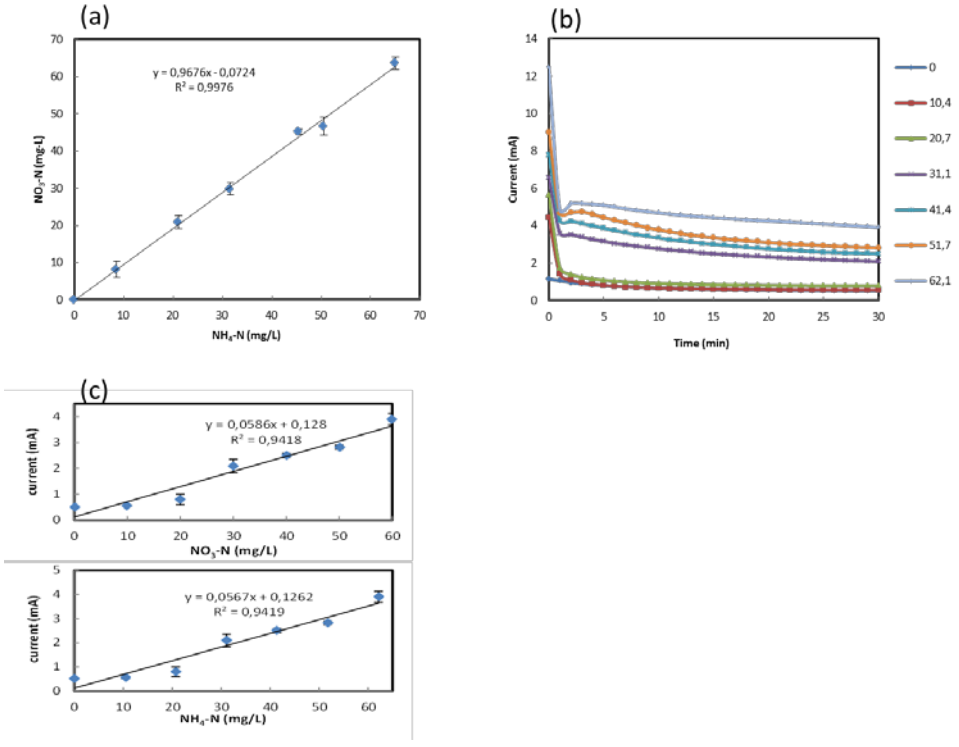

Figure 7. The correlation between the initial ammonia and the accumulated nitrate concentrations in the nitrification process (a). The circuit current of the MEC fed with the nitrification effluent in the cathode in batch mode (b) and the linear relationship between current and ammonia concentrations at external power supply of 0.8 V and pH 6 (c). (Zhao et al. 2018b)

2.2.3 Effect of external power supply and pH on biosensor performance

Similar to the EC-based biosensor, the applied voltage and pH are also important for evaluating the stability and performance of this MEC-based biosensor. As shown in Fig.8a, the linear relationship was always observed for the ammonia and current signals regardless of applied voltages. The correlation coefficient increased with applied voltage. It could be explained by the accelerated electron transfer kinetics at elevated voltages (Perez and Bisang 2016), which directly moved forward the nitrate reduction in MEC cathode. The current increase was more pronounced at higher ammonia levels, which may indicate the voltage was a limiting factor at higher range of ammonia concentrations. On the contrary, at low ammonia levels, the nitrate derived from ammonia oxidation was the limiting factor. In the real application, the considerations related to the anodic reaction should be made. For example, high concentrations of organics in waste and high coulombic efficiency of anodic compartment would reduce the requirements of external power.

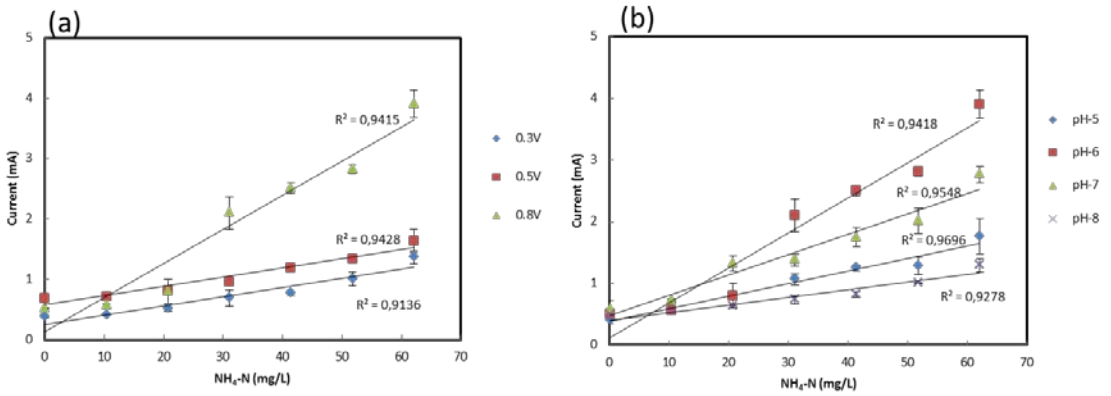

Figure 8. (a) Effect of external applied voltage on the performance of ammonia biosensor. The initial pH of the nitrification effluent is 6 in the cathode. (b) Effect of pH on the performance of ammonia monitoring at external applied voltage of 0.8 V. (Zhao et al. 2018b)

The electrocatalytic nitrate reduction in MEC cathode was reported to be affected by the pH conditions (Zhang and Angelidaki 2012c). Here we applied pH 5,6,7,8 to investigate the effect of pH on biosensor performance. As depicted in Fig.8b, a linear relationship was obtained at all pH conditions, suggesting its stability. However, the slopes were different among these conditions. A higher slope was obtained at lower pH, which might be due to the accelerated nitrate reduction at acidic environment (Abdallah et al. 2014). From the reaction below, the proton was involved in the nitrate reduction,

which meant high concentration of protons would stimulate a faster reaction rate when the nitrate concentration was enough.

$$NO_3^- + 6H^+ + 5e^- \rightarrow \frac{1}{2}N_2 + 3H_2O$$

The good performance of sensor at various applied voltages and pH conditions indicated it was a robust, reliable and applicable biosensor.

2.2.4 Application in real waste streams

Table 3. Determination of NH_4^+-N in real waste streams by the biosensor and commercialized ammonia testing kit. (Zhao et al. 2018b)

sample	NH_4^+-N[a] (mg/L)	NH_4^+-N[b] (mg/L)	pH	Conductivity (us/cm)
AD effluent	896.50 ± 7.78	913.14 ± 12.63	8.73± 0.10	1258 ± 8
Effluent from MBR	45.95 ± 1.06	50.00 ± 1.41	6.27± 0.10	1932 ± 5
Domestic wastewater	20.35 ± 0.07	13.61 ± 1.17	6.54± 0.10	2120 ± 4

[a]Measured by testing kit.

[b]Measured by biosensor.

To prove its real applicability, several types of real waste streams were monitored. The results were compared to that tested by the testing kits, as displayed in Table 3. Anova was conducted to do the statistical analysis between two series of data. The results exhibited no significant difference ($P = 0.98 > 0.05$) at 95% confidence level, demonstrating a reliable result given by the MEC-nitrification biosensor. The detection range could meet the monitoring needs for common waste streams and convincible results with dilutions.

2.2.5 Significance and outlook of this technology

The simultaneous carbon and nitrogen removal was previously demonstrated in the bioelectrochemical-nitrification system. In this work, we expanded its application niche for online ammonia monitoring. Compared to the conventional popular ion-selective electrode ammonia sensor or testing kits, this integrated MEC-nitrification process has several merits. First, it doesn't need any extra space or reagent, and realize a true environmentally friendly platform. Secondly, the in situ monitoring greatly reduces the complexity of sampling work. Thirdly, it's time-saving compared to the APHA method. Finally, using current as signals is more straightforward and easy to operate. The great profit of this integrated system was to monitor the ammonia when removing the carbon and nitrogen at the same time. Besides, this work pro-

vides another alternative approach to the present ammonia detection systems with reliable, cost-effective, time-saving and sustainable advantages.

Although promising, there're still several challenges which should be addressed to further improve the system. Firstly, simplify the configuration of MEC-nitrification set up, for example making nitrification and denitrification occur in a same cathode chamber instead of two separate chambers. Secondly, shorten the response time when large quantities of samples are needed. The time could be reduced via fabricating or modifying the electrode materials. Thirdly, long-term operation should be demonstrated for future commercialization. Last but not the least, maintaining an effective biocatalyst with regard to a thick and electrochemically active biofilm was deciphered as a determining factor to promote the working efficiency of MEC-based ammonia biosensor.

3 Anaerobic granular sludge (AGS) as novel biocatalyst

In recent years, MET-based technology, in particular, MFC is employed for wastewater treatment coupled with electricity generation by exploiting electroactive bacteria to degrade organics in wastewater and produce electrons (Liu et al. 2004, Min and Logan 2004, Rabaey and Verstraete 2005). Due to the renewable and environmentally friendly features, MFC has attained more and more attentions in wastewater treatment field. Though promising, MFC technologies still have several drawbacks such as low CE, fragile to external environment shock and thin biofilm resulting in low organic removal rate. Thus, how to develop a thick and efficient electrochemical biofilm is meaningful to promote the performance of MFC. On one hand, thick biofilm and high biomass would lead to high capacity of organic removal and thus reduce the extra post-treatment cost compared to the conventional inefficient MFC technology (Ren et al. 2014, Wang et al. 2011). On the other hand, efficient electroactive microorganisms would lead to substantial quantities of energy to compensate the practical energy consumption (Lovley 2006a, Zhang and Angelidaki 2016).

Anaerobic granular sludge (AGS), as aggregates of microorganisms, is popular as anaerobic biocatalysts for biogas production and wastewater treatment in the past decade. Due to the dense microbes and granular structure, it is capable to remove the organics and tolerant to extreme conditions (Lettinga and Pol 1991, Schmidt and Ahring 1996). Previously, He et al. (He et al. 2005) demonstrated the feasibility to utilize the ground and filtered AGS as inoculum for powering up an MFC, and high organic removal was achieved. However, low CE was observed, indicating the active methanogenesis in the organic degradation process. This study provided strong proof that AGS could function as the electroactive source. Based on the aforementioned hypothesis, in this study, intact AGS was investigated for its feasibility to treat wastewater in MFC. To achieve this goal, three strategies were conducted to shift the AGS from methanogenic to exoelectrogenic, and organic removal and CE was studied to characterize its performance. Finally the microbial community was analysed to provide microbiological support for the effective strategy.

3.1 Strategies of turning AGS from methanogenic to exoelectrogenic

In paper III, AGS, which was collected from a mesophilic upflow anaerobic sludge blanket, were filled in the anode chamber of an MFC. The MFC was composed of one anode chamber and cathode chamber, which were separated by a cation exchange membrane (CEM, CMI 7000, Membrane international, NanJing). A carbon brush wound into two twisted titanium wires (5.0 cm diameter, 5.0 cm length, Mill-Rose, USA) and a titanium woven wire mesh (4×4 cm, 0.15 mm aperture, William Gregor Limited, London) coated with 0.5 mg/cm^2 Pt were used as anode and cathode respectively. Ag/AgCl electrode (+0.197 V vs SHE) as a reference electrode was placed close to anode. All the values reported here is versus Ag/AgCl. The artificial wastewater was prepared with sodium acetate as described before (Zhang et al. 2011b).

Table 4. Operational time schedule reagrding to three strategies

Time (d)	Experimental condition	
0-27	R-1000, 1.5 g/L	Strategy 1
27-44	R-10, 1.5 g/L	
44-53	R-10, 1g/L	Strategy 2
53-62	R-10, 1.5g/L	
62-69	R-10, 3g/L	
69-83	1g/L	ChronAmperometry,Strategy 3
83-98		Charge-discharge experiments
98-108	1g/L	After potential control, AGS-MFC
108-129	1g/L	Moving granules out, Control 1
129-138	1g/L,pH-5	Moving granules back, AGS-MFC
138-147	1g/L,pH-6	
147-158	1g/L,pH-7	

During the acclimatization of methanogenic AGS in exoelectrogenic conditions, three strategies were employed continuously (Table 4). After the measurements of organic removal, current generation and biogas composition, significant positive effects were obtained in Strategy 3 (anodic potential controlled at +20 mV). When the external resistance was varied from 1000 Ω to 10 Ω, both the organic removal (from 67.15% to 86.85%) and current density output (maximum current density increased from 0.41 to 5.84 A/m^2) was increased (shown in Fig.9A and C). This suggests the anaerobic substrate oxidation was enhanced at lower resistance, which might be ascribed to the anaerobic methanation (indicated by significantly higher methane production in

Fig.9E). The results were different from the previous observations in other studies, which show that methanogens were suppressed at lower resistance (Butti et al. 2016, Jung and Regan 2011, Song et al. 2010). It could be explained by the faster interspecies electron transfer between exoelectrogens and methanogens (Lovley 2011) accelerated by reducing external resistance. Considering the improved electricity generation, R-10 Ω was chosen for the Strategy 2 operation. In Strategy 2, to fully inhibit the methanogenic activity, organic loading was manipulated, varying from 1000 to 3000 mg/L. As depicted in Fig.9A and C, the current wasn't elevated along with organic concentration increasing whereas COD removal rate was significantly increased with organic loading increasing. The promotion of COD degradation was mainly derived from the methanation process. The deduction was supported by the biogas composition and methane yield analysis (Fig.9E). Overall, the above results demonstrated that the acetate was not the limiting factor for promoting exoelectrogenic activity and suppressing methanogensis activity.

In order to realize the two sub-objectives of activating exoelectrogens and inhibiting methanogens, controlling anodic potential at positive value (+20 mV) was employed as Strategy 3. It has been widely reported that anodic potential is positively associated with current generation and microbial community in MFC anode (Torres et al. 2009, Wagner et al. 2010, White et al. 2009). Theoretically, exoelectrogens could gain the energy for their growth and maintenance according to the equation (Schroder 2007):

$$\Delta G^{0'} = -nF(E_{anode} - E^{0'}_{donor})$$

$\Delta G^{0'}$ is the Gibbs free energy change at standard conditions (pH 7 and 25°C), n is the number of electrons transferred, F is Faraday's constant (96485 C mol^{-1} e^-), E_{anode} is the anode potential, $E^{0'}_{donor}$ is the standard biological redox potential of electron donor. From the equation, we know that at higher anodic potential, more energy would be released. When the anodic potential was elevated from -500 mV to +20 mV, more energy would be utilized by the microorganisms and thus accelerating the electron hopping from cells to anode or electron transport within biofilm. In this manner, the acetate utilization was promoted and the corresponding current generation was greatly improved (Fig.9B and D). Notably, the methane production was close to 0 under positive anodic potential, suggesting the suppressed methanogenic activity at higher anodic potential.

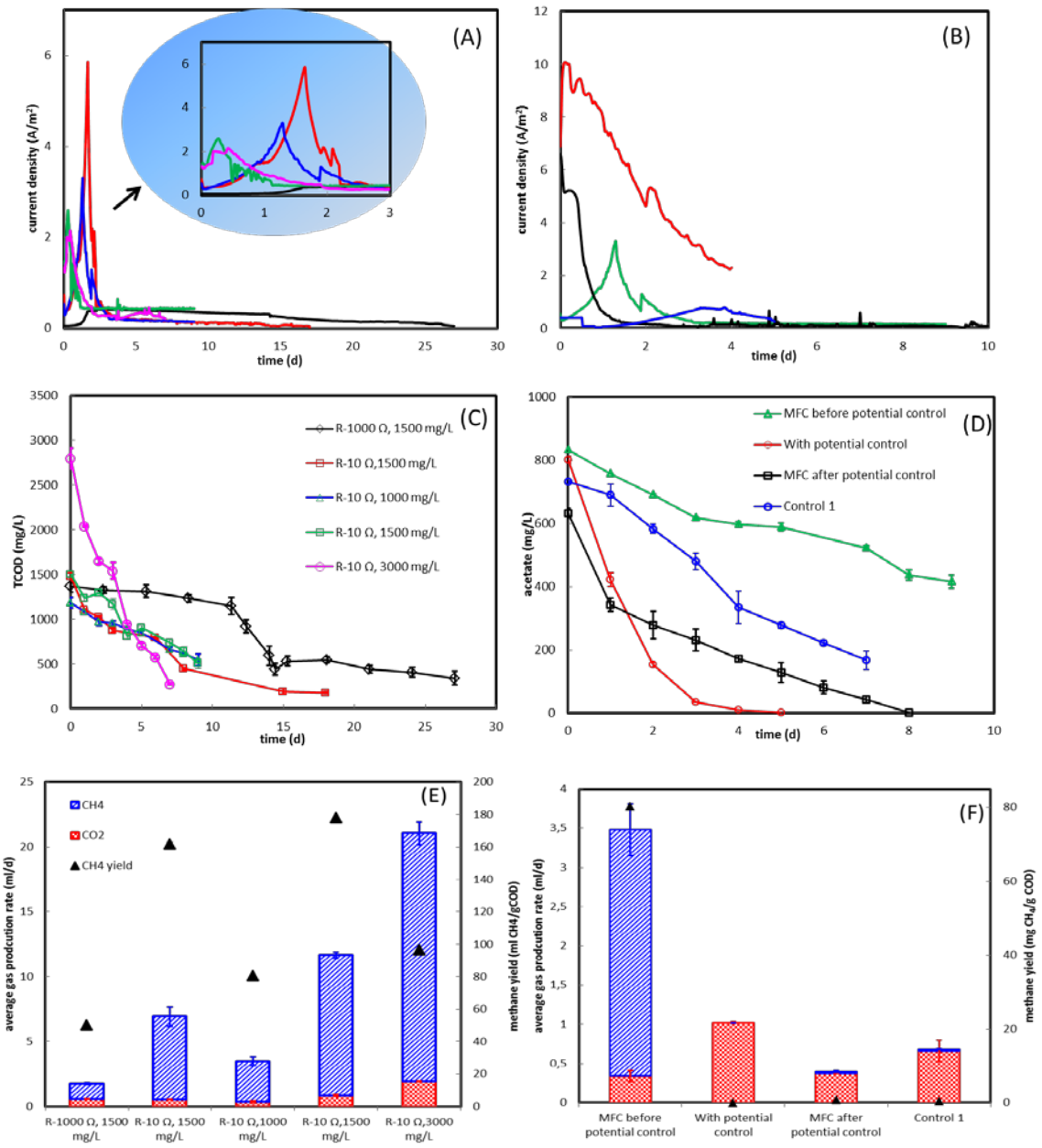

Figure 9. Current response in Strategy 1 and 2 (A) and in Strategy 3 (B); organic concentration in Strategy 1 and 2 (C) and in Strategy 3 (D); methane yield in Strategy 1 and 2 (E) and in Strategy 3 (F). Strategy 1: external resistance change from 1000 Ω to 10 Ω; Strategy 2: organic loading vary from 1000 mg-acetate/L to 3000 mg-acetate/L; Strategy 3: anodic potential control at +20 mV. Control 1: MFC with only bio-carbon brush (after anodic potential control and moving granules out).

To evaluate the effect of this strategy on long-term operation, the reactor was subsequently switched to MFC mode without anodic potential control (Fig.9B and D). Clearly, the peak current density increased from 3.30 (before potential control) to 6.41 A/m^2 (after potential control) in the same acetate concentration (1000 mg/L), and acetate removal efficiency increased from 50.12%

(9 days) to 100% (8 days). The results indicated the positive anodic potential control was effective to have a persistent effect on the long-term running regarding to the exoelectrogens enrichment. In terms of the inhibited methanogens, it was observed the methane production wasn't recovered back compared to the MFC before Strategy 3. In order to differentiate the contribution of AGS in electricity generation and carbon removal, the MFC performance was investigated when all the granules were removed out (Control 1). The immediate decreasing of current peak values from 6.59 to 0.52 A/m^2 suggested an active and vital role of AGS in the electron transfer. The according acetate removal efficiency was 77.3%. Since no methane production was tested in this case, it was plausible to ascribe the organic removal to electrocatalytic degradation. When removing all AGS from the anode, it could be that residual AGS or biofilm attachment was still present, contributing to the small amounts of electricity generation.

Taken together, promoting anodic potential was deciphered as an effective way to shift the methanogenic AGS to exoelectrogenic for simultaneous effective organic removal with current generation.

3.2 Robust to acid conditions

In the conventional MFC, the domestic wastewater was often used as inoculum to enrich the electroactive biofilm (Ren et al. 2008). It's either attached on anode or suspended in liquid, which was reproted to be sensitive and fragile to acid conditions (Du et al. 2017, Leong et al. 2013). Comparatively, the diverse microbes and intrinsic granular structure of AGS enabled it resistant to extreme environments (Daud et al. 2018, Tan et al. 2018). Here, we investigated its resistance to acid pH.

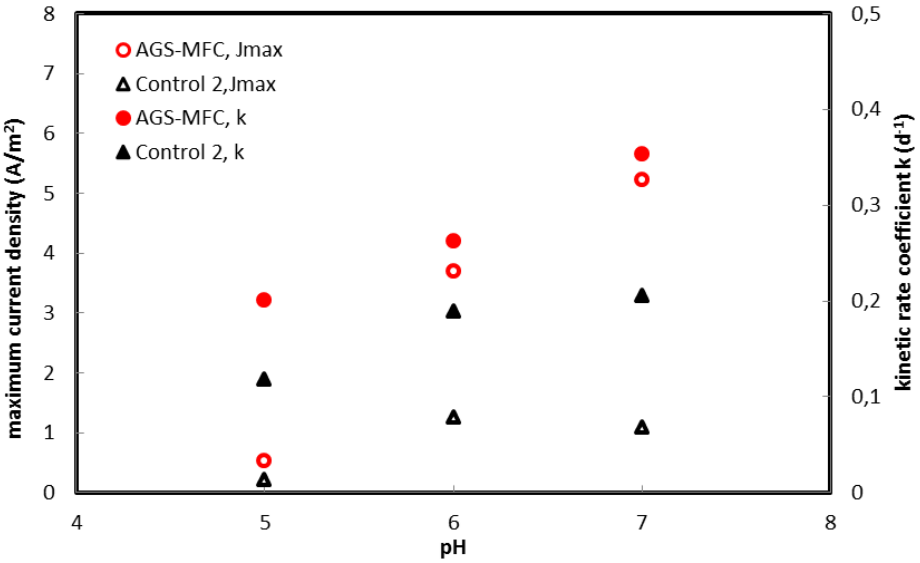

Figure 10. The maxmium current density and COD removal rate coefficient at varied pH conditions. AGS-MFC: MFC after potential control; Control 2: MFC inoculated with domestic wastewater.

Based on the current generation and acetate removal rate, we plot the data of maxmium current density at each condition in Fig.10. And based on the assumption of first-order kinetics, the rate coefficient was calculated accordign to the equation

$$Ln\frac{COD_t}{COD_0} = -kt$$

where COD_0 is the initial COD concentration, COD_t is the COD concentration at time t, and k is the first-order kinetic rate constant.

As shown, compared to conventional MFC inoculated with domestic wastewater, at each pH, maxmium current density was higher in granular MFC, indicating its good tolerance to acid conditions. The kinetic rate coefficient showed a similar manner to pH variations. Higher kinetic rate coefficient suggested faster organic degradation in granular MFC in all the tested pH. It has to be mentioned that at lower pH, both reactors performance was indeed affected, indicated as the decreasing maximum current density and kinetic rate co-efficient. But notably, the rate coefficient of granular MFC at pH 5 was close to the level of that in conventional MFC at pH 7. It meant a superior/stable performance of granular MFC even at unfavourable pH conditions. Obviously, from the pH resistance experiments, neutral pH has been proved to be the optimal condition for the exoelectrogenic bacteria.

3.3 Morphological characteristics and elemental composition of AGS

According to the phenomenon previously described, we deducted that the granular structure was one of the key factors playing an important role. Therefore, to convince, we took the granules after strategy 3 for morphological characteristics, as shown in Fig.11.

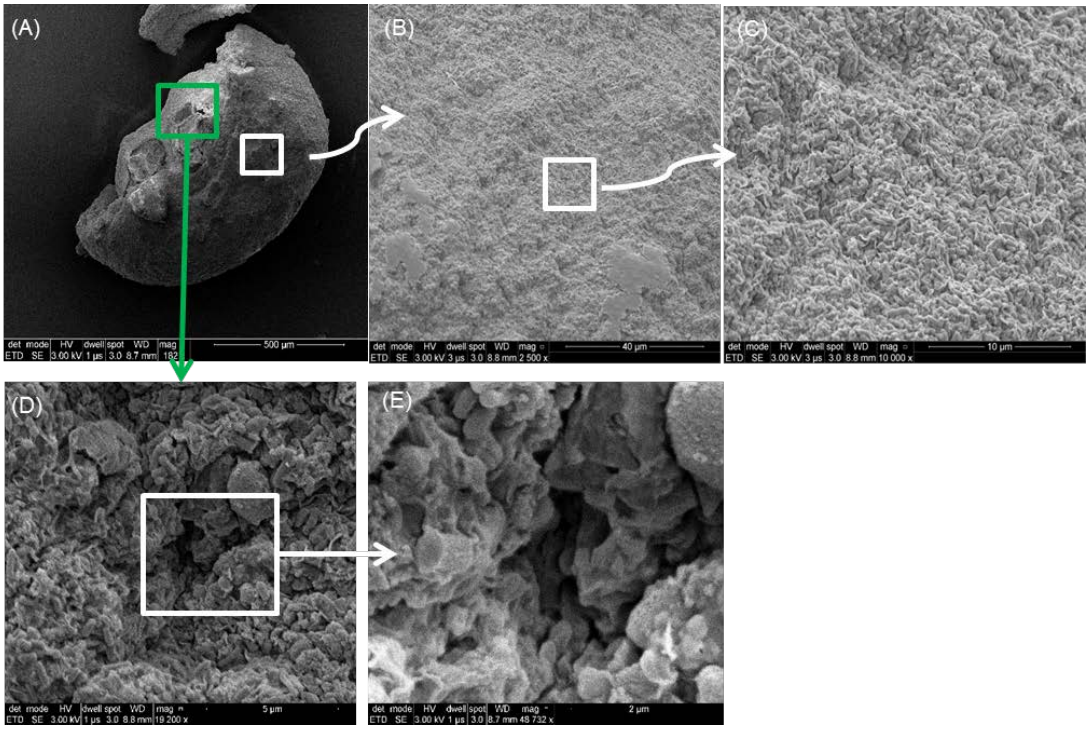

Figure 11. SEM image of the surface struture of single GAS after anodic potential cotnrol. (A) an intact granule; (B) and (C) high-resolution of SEM image of granular surface showing the massive microbial colonization; (D) and (E) showing the rod-shape mircobes aligned on the side of deep channels.

The single AGS has spherical rough surface and macro-porous carbon architecture. A zoomed in image of the surface is shown in Fig.11B and C, in which the entire surface was occupied by the rod-shape bacteria. The rod-shape bacteria were often found in the microbial community of MFC (Sevda et al. 2013, Torres et al. 2009). The porous structure, as verified by the multi-channels (Fig.11D and E), had several advantages: 1) it was beneficial for the substrate exchange in and out to maintain a good mass transfer; 2) it provided large surface area for biofilm attachment and growth. Moreover, the granular structure, compared to the conventional flocs-style biofilm, was more resist to the external disturbance of the surrounded environments (Quarmby and Forster 1995). One more thing needs to be noticed was that after the anodic

potential control, the granular structure was still intact, indicating a robust property of the granules.

3.4 Microbial community dynamics

After the selection and comparison of three strategies, strategy 3 was demonstrated as the most effective to improve the current generation and inhibit methane production. To further confirm our deduction, 16S rRNA was employed to take a deep look into the microbial community dynamics in granules and biofilm before and after the strategy 3.

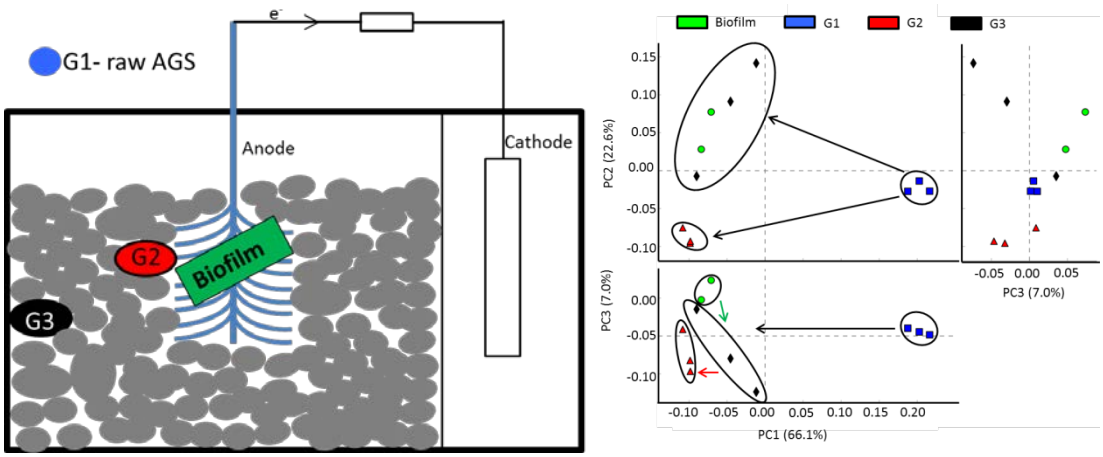

Figure 12. The schematic of the sampling sites (left) and Beta diversity of triplicate samples in MFC (right). It includes enriched biofilm on carbon brush (Biofilm), raw AGS (G1), enriched AGS taken close to carbon brush (G2), and enriched AGS taken far from carbon brush (G3). Principal components (PC) 1, 2 and 3 explained 66.1%, 22.6% and 7%, respectively. The black arrow indicated a dramatic change between G1 and G2/Biofilm/G3 based on the principal percentage (PC1 and PC2). The red and green arrow indicated distinct difference between G2 and G3, and between granules and anodic biofilm, respectively.

The sampling sites were depicted in Fig.12 (left) and the beta diversity results were shown in Fig.12 (right). As shown, a distinct microbial dynamic change was observed before and after anodic potential control. Based on the principal positions (PC1 and PC2), it was noticed that a dramatic change between the raw granules and acclimatized granules and biofilm. Based on the further analysis of PC3, the granules taken from different positions showed difference to each other, and the biofilm also show same manner to the granules. The above results were in accordance with the previous studies that the microbiome clustering was changed by changing anodic potential (Hari et al. 2016).

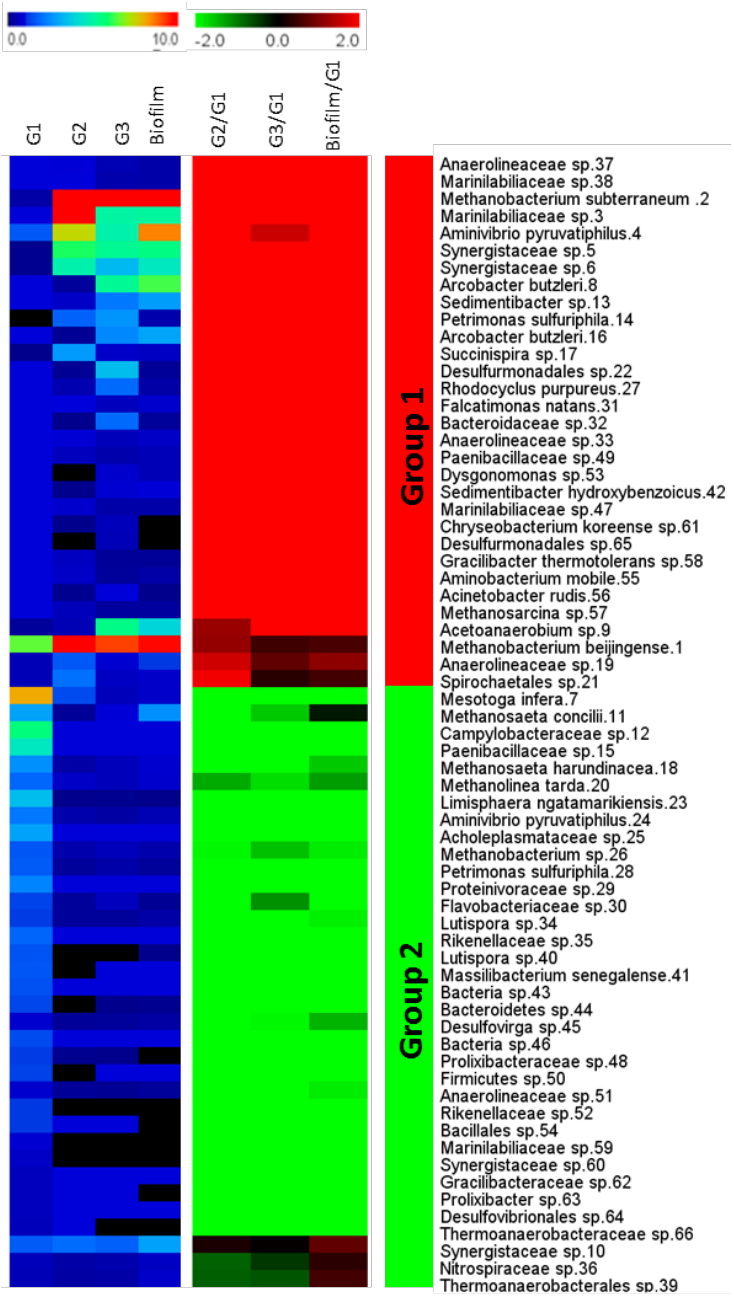

Figure 13. Microbial community compositions in raw AGS (G1), and enriched AGS after anode potential control and close to carbon brush (G2), enriched AGS far from carbon brush (G3), and biofilm on carbon brush (Biofilm). Relative abundance (%) and folds change were reported in (A) and (B), respectively. Group 1: the taxa increased in relative abundance after anode potential control. Group 2: the taxa decreased in relative abundance after anode potential control.

In order to analyse microbial dynamics in the granules and attached anodic biofilm, high throughput 16S rRNA amplicon sequencing was employed. Only the relative abundance of taxa over 0.5% was selected as interesting species, as shown in Fig.13. The majority of microbial community in raw gran-

ules was bacterial, accounting for 90%. The vast innate bacteria groups enable the granules as an ideal inoculum source.

The changes between raw granules and enriched ones after anodic potential control were evaluated and presented in Fig.13B. As displayed, group 1 was significantly increasing in relative abundance after anodic potential control. The vast majority of this group was associated to exoelectrogens. For example, the relative abundance of *Synergistaceae* spp. (5 and 6) showed a significant increase from 0.2% to over 10% in the enriched granules and biofilm, which could be explained by the positive anodic potential. This family *Synergistaceae* was often deciphered in MFC anode. It is worthy to pay attention to the *Desulfurmonadales* spp., which has been well known for its function of active role in electrochemically acetate degradation and electron transfer (Borole et al. 2011, Zhang and Angelidaki 2012a, Zhao et al. 2017), appeared after the anodic potential control. The *Desulfurmonadales* spp. (22 and 65) showed a 97% similarity to *Pelobacter propionicus* and *Geobacter chapellei*. Although the main function of *Pelobacter propionicus* was to produce propionate from acetate, no propionate was monitored in this study. Therefore, it's reasonable to affiliate this strain to *Geobacter chapellei*, which was known as ferric reducer (Liu et al. 2013). Since the iron-reducing bacteria are involved in the direct electron transfer from cells to electrode, it's deduced that the *Geobacter chapellei* we found here may also use electrode as electron acceptor to complete a direct electron transfer pathway (Logan and Regan 2006). Furthermore, the strain *Marinilabiliaceae* spp. (3 and 47), which were often previously found in the electrochemical bacteria community in MFC anode (Sotres et al. 2016), also showed an obvious increase in the relative abundance.

On the contrary, another cluster of taxa showed a significant decrease in the relative abundance, indicating the transition from negative anodic potential (-500 mV) to positive anodic potential (+20 mV) set a resident niches to these taxa. Specifically, *Mesotoga infera,* known as the acetate oxidizing bacterial (from acetate to CO_2 and H_2) (Ahlert et al. 2016), showed a decrease from 9% to 0%, indicating this strain was totally suppressed at positive anodic potential. Similarly, *Methanosaeta concilii,* which was reported to be capable to transfer the electrons with *Geobacter* species for further CO_2 reduction (Holmes et al. 2017), decreased from 2.89% to 0.15%. It suggested the interspecies electron transfer pathway was blocked with elevating anodic potential. As reported, the potential for maintaining methanogens growth should be

less than -527 mV (vs SHE) (Lange and Ahring 2001). This simply explained the suppression of methanogens at +20 mV.

To conclude this work, we could propose an effective strategy of controlling anodic potential at positive values to successfully shift the methanogenic AGS to exoelectrogenic AGS, and further employ the exoelectrogenic AGS for effective carbon removal in the wastewater. The morphological and microbial analysis supported the deduction that methanogens were suppressed whereas exoelectrogens were activated.

It has to be mentioned that from the later research work (paper IV), it would be interesting to exploit the electrochemical approach (i.e. cyclic voltammetry) to characterize the exoelectrogens colonization.

4 Electron storage capability of AGS

High energy demand drive by the increasing population and urgent requirements of carbon-neutral society points to two directions: one is to develop innovative technology to use renewable energies (Demirbas et al. 2011) and the other is to develop alternative energy storage device (Saar et al. 2018). One the one hand, regarding to the renewable sources, biomass is more attractive and competitive since it's independent of seasons or countries while the wind power or solar energy varies with seasons or daylight time. MET, as introduced in chapter 1, is an innovative new technology to extract energy (i.e. electricity or desirable products) from biomass with the help of exoelectrogens (Lovley 2006b). On the other hand, biomaterial-based capacitor has emerged as an attractive energy storage device (Ren et al. 2015). It has been widely reported the protein structure (i.e. cytochromes) widely present in exoelectrogens have the capacity to store electrons (Agnès et al. 2014, Lu et al. 2015, Malvankar et al. 2012, Ren et al. 2015). There have been some publications to fabricate the electrode with cytochromes to employ as a biocapacitor (Bonanni et al. 2012, Deeke et al. 2012). Taking together, MET-based capacitor develop would benefit not only the renewable energy utilization, but also the electron storage device.

Recently, there's one publication to investigate the electron storage in one exoelectrogenic activated carbon granule (Borsje et al. 2016). Inspired by it, an AGS-based capacitor was proposed, and aimed to produce electricity from biomass first, and then conserve the electrons temporarily. In that case, we combine the energy production along with energy storage together in an environmentally friendly way. One more thing needs to be mentioned is that when we develop a AGS-based biocapacitor, we not only exploit the electron storage capacity of exoelectrogens, but also make the use of double electrical layer effect, which is the main principles of conventional electrical capacitor (Beguin et al. 2014, El-Kady et al. 2016). The concept of AGS-based biocapacitor opens a new pathway towards combination of biology, electrochemistry and electronics science.

4.1 Electrochemical characterization of AGS

To investigate the capacitance of exoelectrogenic AGS, first question is to turn the raw AGS (methanogenic) to exoelectrogenic. As stated in Chapter 3, controlling anodic potential was demonstrated as an effective approach to shift the methanogenic AGS to exoelectrogenic. Thus, here to get an electroactive AGS, we employed the chronoamperometry, which meant anodic po-

tential controlled at +0.2 V, to the developed AGS-based biocapacitor. The current generation profile as shown in Fig.14a showed a typical trend in the batch operation. Maximum current (around 40 mA) was obtained after 7 days' operation, and then decreased rapidly due to the depletion of substrate. The repeatable cycles indicated a mature electroactive biofilm formed on anode. To further confirm our deduction, polarization curve and CV test were performed, as shown in Fig.14b, c and d. In the polarization test, obviously the exoelectrogenic AGS showed significantly higher current response compared to the raw/methanogenic AGS and abiotic electrode during all the scanning potential range. Similarly, from CV characterization, exoelectrogenic AGS showed a quite typical Nernstian sigmoidal shape, which was a representative for electrocatalytic species in MFC (Du et al. 2017, Fricke et al. 2008). All the electrochemical characterization results proved the AGS was electroactive after the chronoamperometry.

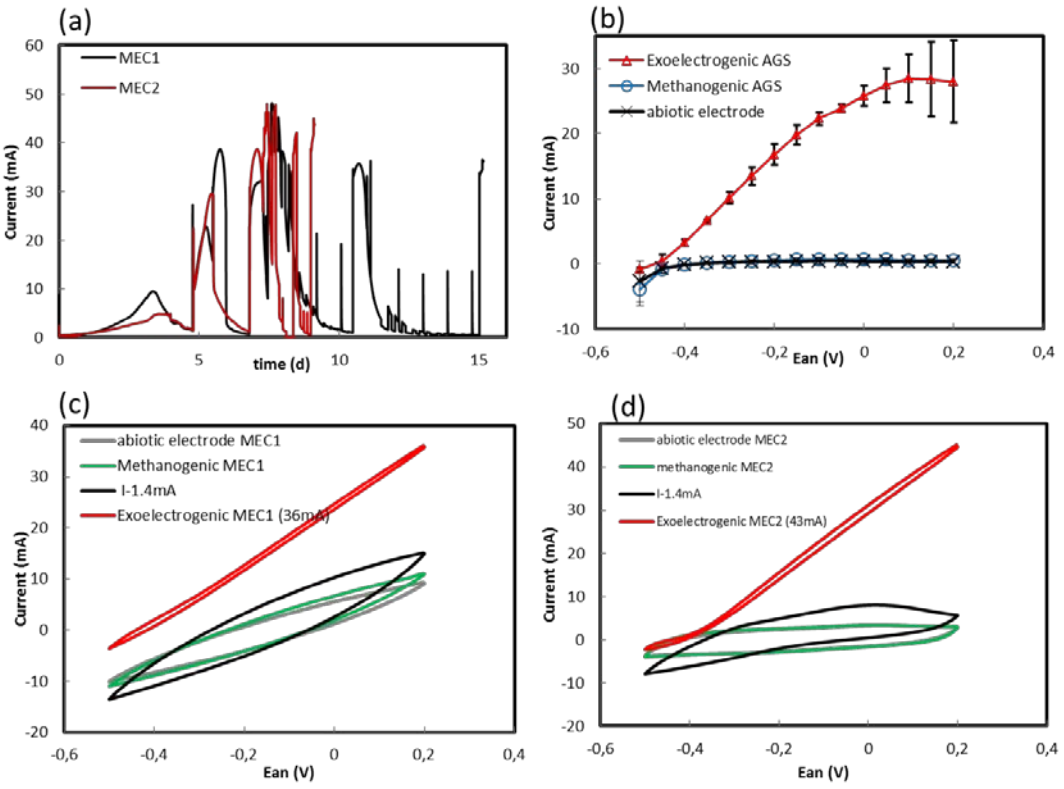

Figure 14. Electrochemical characterization profiles of exoelectrogenic AGS at a carbon brush electrode with 10 mM acetate as substrate. (a) Chronoamperometric plot of the formation and bioelectrocatalytic activity of exoelectrogens in AGS, (b) polarization curve of electrode and AGS at different stages, (c) cylic voltammogram of the exoelectrogenic AGS-MEC1, and (d) cylic voltammogram of the exoelectrogenic AGS-MEC2.

4.2 Multi-parameter optimization of capacitive performance

As stated in the literature, cycle test (charge-discharge) is an appropriate approach to investigate the capacitance of a biocapacitor (Borsje et al. 2016, Deeke et al. 2012). Charge referred to the open circuit mode while discharge referred to the controlled anodic potential at 0 V (Fig.15). The anode potential of 0 V was chosen, because at this applied potential the current density reached a plateau as what have been indicated in polarization curve. If a system has the ability to store electrons, the current should be composed of capacitive current and faradic current.

The representative cycle test profile of the AGS-based system was displayed in Fig.15. As depicted, when the electrical circuit was open, the anodic potential showed an immediate decrease, as similar to the previous finding (Borsje et al. 2016). The potential drop is dictated by the accumulated electrons on the anode. Once the circuit was closed, the anodic potential was artificially corrected at 0 V, and all the accumulated electrons during charging process were conducted through the circuit. The current profile showed typical capacitive features. The area of peaks referred to the amount of capacitive current, which indicated the amount of energy stored in anode. The stable current referred to the faradic current, which was related to the electrons released from acetate oxidation. According to the mathematic calculation by integral of current with time, the cumulative charge (Qcum), stable charge (Qst) and capacitive charge (Qcap). Qcap is an important parameter to indicate the storage capacity of a capacitor. Normally, the higher, the better. Qcap for MEC1 and MEC2 were 2.11 and 2.66 C, respectively. The values were comparable to the Qcap of methanogenic AGS (0.015 ± 0.045C) at same condition. The great improvement was ascribed to the enrichment of exoelectrogens in AGS. The primary functional structures of electron storage in exoelectrogens were reported to be the phosphor-lipid bilayer structure, c-type cytochromes and nanowires (Ren et al. 2015, Strycharz-Glaven et al. 2011a). Therefore, the cycle test performance fit to our expectations of the new biocapacitor.

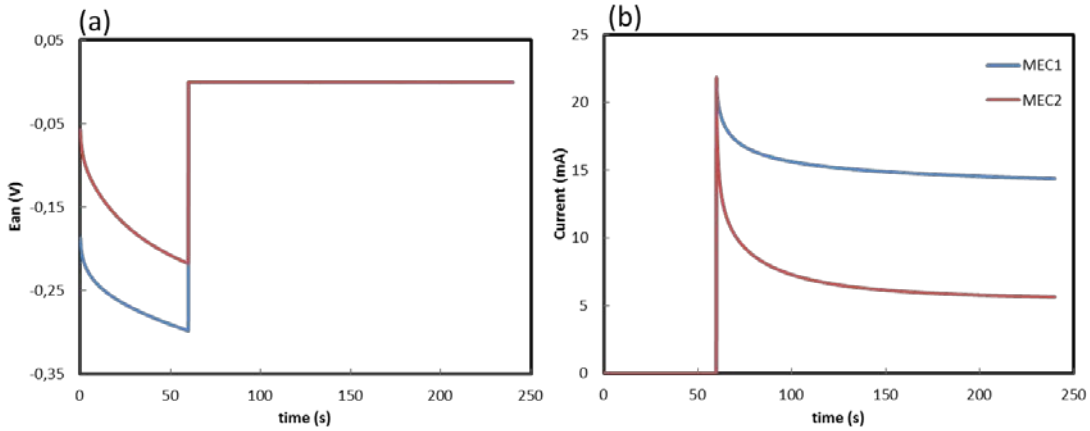

Figure 15. Typical charge-discharge test at 0 V. Anodic potential change (a) and current response during a single cycle of 60 s charge and 180 s discharge period.

To optimize the capacitive performance of this new biocapacitor, we employed several test to see the effects of these parameters on the performance. All the cycle tests were performed for 10 consecutive times. Based on the current profiles, the corresponding Qcap was calculated as displayed in Fig.16. Clearly, Qcap increased with anodic potential, which was ascribed either to the increasing activity of exoelectrogens with elevated anodic potential (Schroder 2007) or to the higher amounts of proteins at more positive potential (Carmona-Martinez et al. 2013). Since at +0.2 V, the Qcap achieved the highest value. Therefore, in the following test, this value was selected as the anodic potential to maintain a good electroactivity. In terms of the charge-discharge time effect, 5 min - 10 min was selected as the optimal time period for the biocapacitor to get fully charged and discharged. 1 min - 3 min was too short for all the bacteria charged evenly. Too long time didn't increase the Qcap. The independence of time indicated it was a robust capacitor, which may benefit its future commercialization. To further differentiate the contribution of AGS to the capacitance, we removed all the AGS for cycle test. Compared with the Qcap of 20 g AGS, a significant decrease was observed when all the AGS were removed, indicating an important role of AGS in the capacitance. The AGS contained high amounts of biomass, thereby more electron shuttles and cytochromes, which result in the electron conservation. In addition, the special granular structure of AGS may result in the double layer capacitor effect. It would disappear after we removed AGS. To short conclude, the optimized anodic potential was +0.2 V, charge-discharge time was 5 min - 10 min and AGS amounts would be as much as possible.

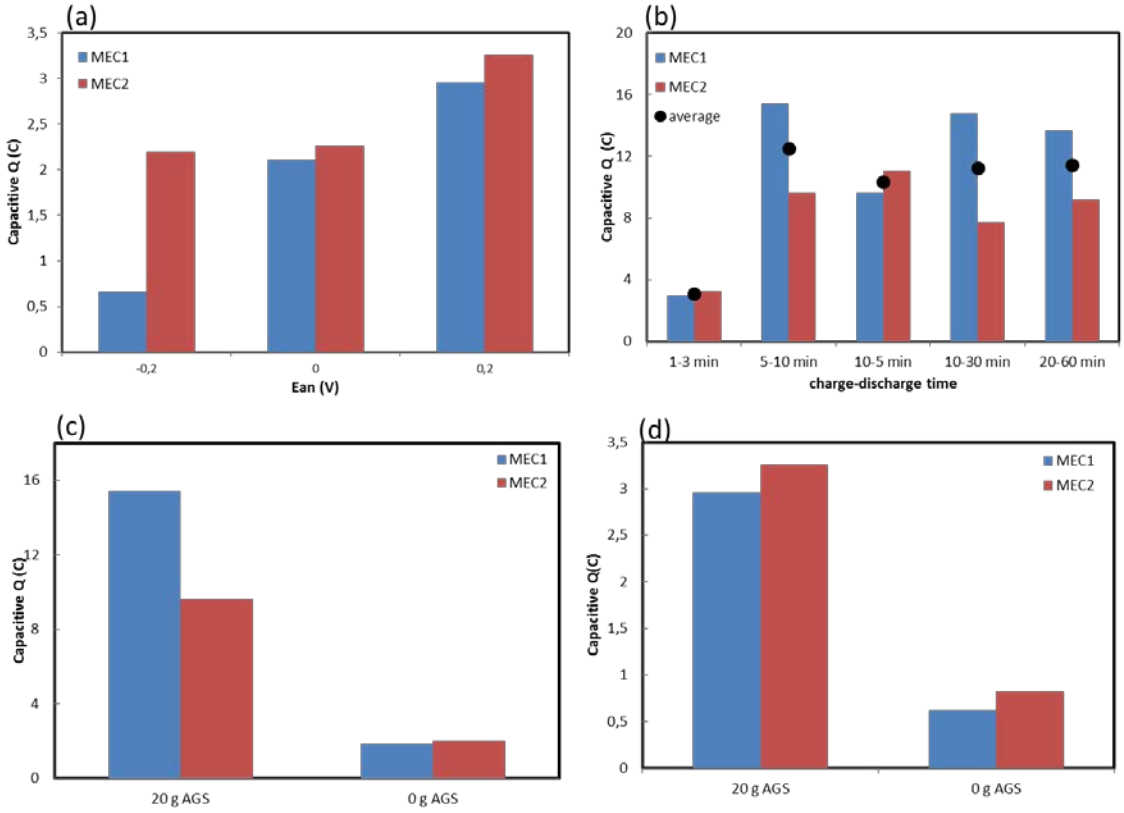

Figure 16. Capacitive Q in multi-parameter conditions. (a) is the capacitive Q of 20 g AGS calculated from 10 consecutive cycle tests operated at different potentials when charge-discharge time is 1 min - 3 min; (b) is the capacitive Q of 20 g AGS calculated from 10 consecutive cycle tests operated at different cycle times when anodic potential was controlled at 0.2 V; (c) is the capacitive Q calculated from 10 consecutive cycle tests operated at 20g AGS and 0 g AGS respectively when anodic potential was controlled at 0.2 V and charge-discharge time was set as 5 min - 10 min; (d) is the capacitive Q calculated from 10 consecutive cycle tests operated at 20g AGS and 0 g AGS respectively when anodic potential was controlled at 0.2 V and charge-discharge time was set as 1 min - 3 min.

4.3 Electrochemical behaviour of single AGS

As stated in section 4.2, the electrons were firstly generated from the acetate degradation via metabolism; secondly the released electrons would be transported from cells inside to outside, then within biofilm matrix and finally electrode. In this transfer process, how the electrochemical communications between granules with electrode would be interesting and helpful to disclose the pathway. This section was divided into two packages. One was to study the electrochemical connections between single granule and flat electrode (i.e. graphite), and the other was to investigate the electrochemical communications between single granule and wrapped electrode (gold wire). The latter work was to increase the connective spots between granule and conductive material.

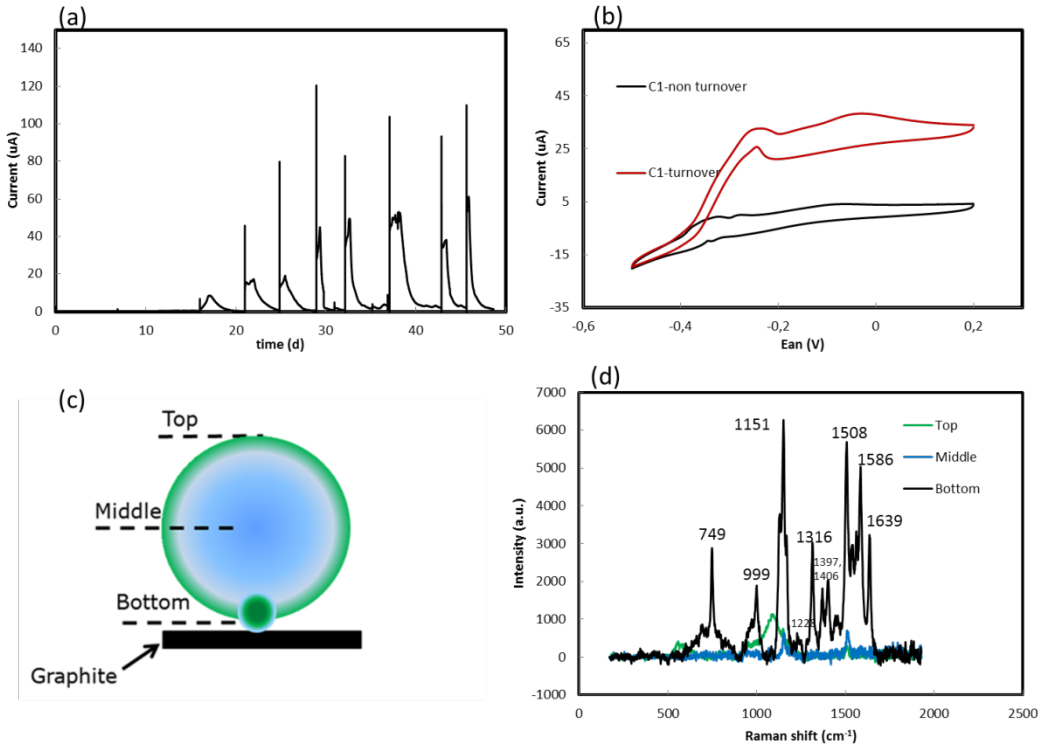

Figure 17. Electrochemical behavior of single AGS growing on the graphite. CA (a), CV (b) profile and sampling sire (c), Raman spectrum (d). CA was performed at +0.2 V.

Fig.17 depicted the results of single granule growing on a graphite electrode (0.20 cm^2). Single granule was sedimented naturally on the graphite, and 10 ml medium was refreshed to achieve a level of 10 mM acetate each batch. Triplicate reactors were setup and here we took one as representative for the following discussion. As shown in Fig.17a, a first electroactivity maximum (indicated as the first I_{max}) was reached, and decreased afterwards due to the acetate exhaustion. After several cycles of medium replenishment, the maximum current of around 50 uA was obtained after 30 days operation of chronoamperometry. CV was employed to provide more information about the electron transfer mechanisms in single granule. Turnover referred to the condition when the acetate was present, while non turnover referred to the acetate depletion. A classical sigmoidal shape was obtained for both the CVs, demonstrating a good electrocatalytic activity. From the mathematic calculation of first derivative curve, the formal potentials were -0.344 V and -0.275 V for turnover CV, and -0.367 V and -0.325 V for non turnover conditions. All these values were in the potential range which was found for the membrane cytochromes responsible for direct electron transfer like OmcB, OmcE and OmcS (i.e. for OmcB, a formal potential of -387 mV vs Ag/AgCl was reported) (Eggleston et al. 2008, Hartshorne et al. 2007). Particularly, the value of -0.367 was quite close to the formal potential (-0.389 V) of *Geobac-*

ter. sulfurreducens, which suggested a *Geobacter* dominated biofilm. The Raman spectroscopy offered supplementary information from the spectrum aspect (Fig.17d). From the spectra, we only observed the characterized peaks of multi-heme *G. sulfurreducens* cytochromes in the "bottom" sampling site (Dick et al. 2000, Hu et al. 1993, Lebedev et al. 2014). The bottom site was considered as the connective spot between electrode and granule. The peaks contained 1) the main heme band at 1586 cm^{-1}; 2) characteristic oxidation state-sensitive bands at 1316 with two additional minor bands at 1397 and 1406 cm^{-1}; 3) strong band at 1508 cm^{-1}, which was associated with heme interaction with a metallic surface (Dick et al. 2000). The above results well distinguished the cytochromes, which were not only participating in the electron transfer chain, but also facilitating in the electron transport via conductive nanowires (Gorby et al. 2006, Inoue et al. 2011). The cytochromes which were only distinguished in the "bottom" site indicated that there was an effective electron flow between granule and graphite.

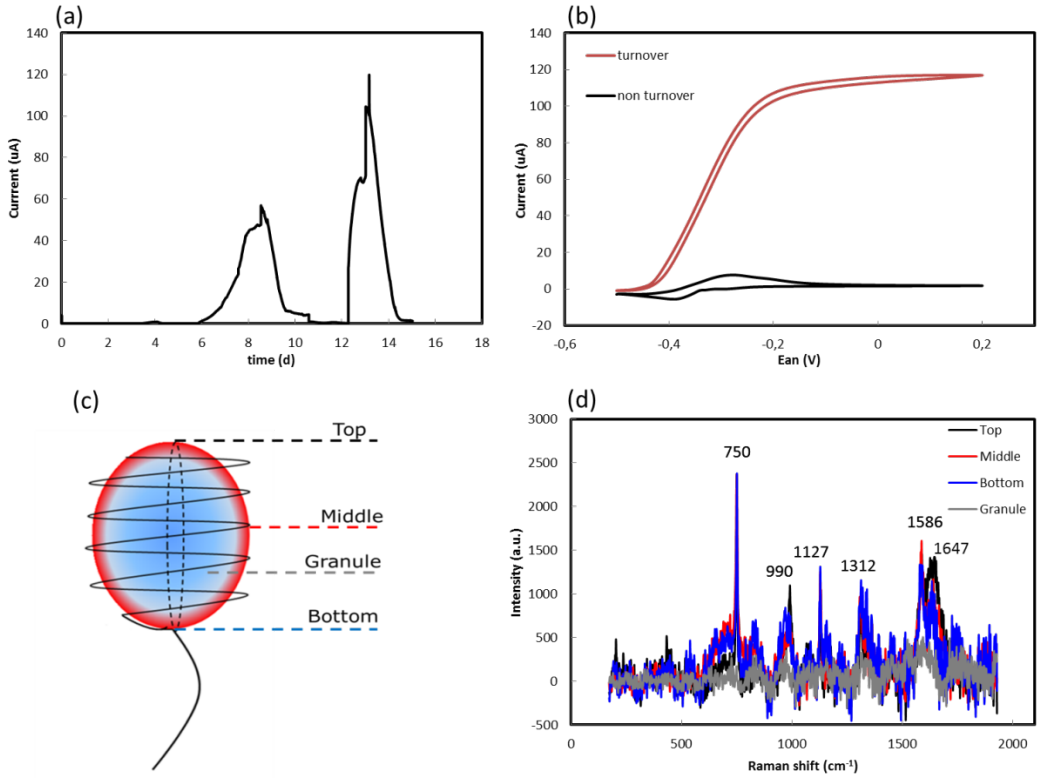

Figure 18. Electrochemical behavior of single AGS wrapped with gold wire. CA (a), CV (b) profile and sampling sites (c) and Raman spectrum (d).

Once the spot of granule was in good contact with conductive material, the spot would be conductive (electron could flow). Thus, to increase the connective spots, we conducted another experiment. We wrapped the single granule with gold wires (Ø 0.1 mm, approximately 5 cm length), and similarly we performed CA and then CV with Raman as well. The CA and CV showed similar manner to the one with graphite (Fig.18). Differently, the Raman spectra exhibited the features of peaks associated with cytochromes in all the sampling sites except the granule sample. It meant all the surrounded spots, which were in good contact with gold wire, were conductive. This undoubtedly supported our hypothesis that more connective spots would result in better electroactivity performance.

4.4 Microbial community dynamics under chronoamperometry

As stated before, the exoelectrogenic AGS outperformed methanogenic AGS was dictated by the active role of exoelectrogens. To confirm the statement, 16S rRNA was used to disclose the microbial community compositions under chronoamperometry. Taxonomic classification of the microbial communities at different levels is displayed in Fig.19a-b (archaea) and Fig.19c-d (bacteria). Regarding to the archaeal compositions, at genus level, the relative abundance of *Methanobacterium* was increased from 53% in methanogenic AGS to over 93% in exoelectrogenic AGS. On the contrary, *Methanosaeta*, known as a unique methanogen exclusively acetate degrader, was significantly decreased from 34% to 1%, suggesting the suppression of acetoclastic methanogens at positive anodic potential. Regarding to the bacterial compositions, it was noteworthy that *Geobacter* accounted for 1.23% in exoelectrogenic AGS. The *Geobacter* was well known for its function to perform direct electron transfer via special structure i.e. conductive nanowire (Adhikari et al. 2016, Reguera et al. 2005, Reguera et al. 2007). The appearance of *Geobacter* suggested that the exoelectrogens were involved in the electron storage process.

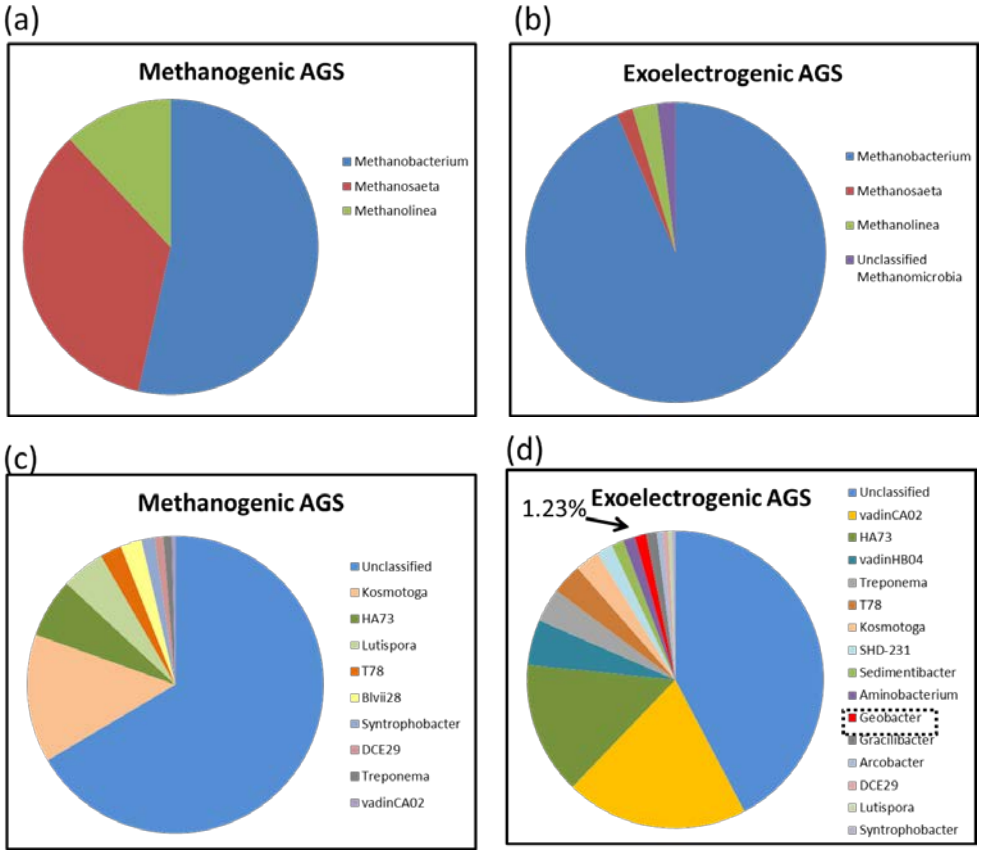

Figure 19. Taxonomic classification of the archaea and bacteria communities (over 0.1% of the relative abundance). The area of pie was based on the relative abundance, which was normalized by the relative abundance affiliated with the taxon divided by the total abundance of sequences per sample. (a) and (b) archaea-genus level; (c) and (d) bacteria-genus level.

4.5 Proposed mechanism of AGS capacitance

Based on the above results, a mechanism of how the biocapacitor store electrons was proposed in this section. To clarify the whole process, it was divided into four steps:

1) At the first beginning, electrons were released from acetate degradation via exoelectrogens metabolism, and the electrons were temporarily preserved in the c-type cytochromes or nanowires;

2) Meanwhile, some of the free electrons were residing at the granule surface, which attracted the cation ions in the electrolyte to maintain electronic neutrality, termed as a double layer effect. In this way, the electrons were stabilized in the granule;

3) Possibly, the biofilm, formed on the electrode, also had the ability to store electrons;

4) A last possibility to preserve electrons was the double layer effect of electrode (carbon brush).

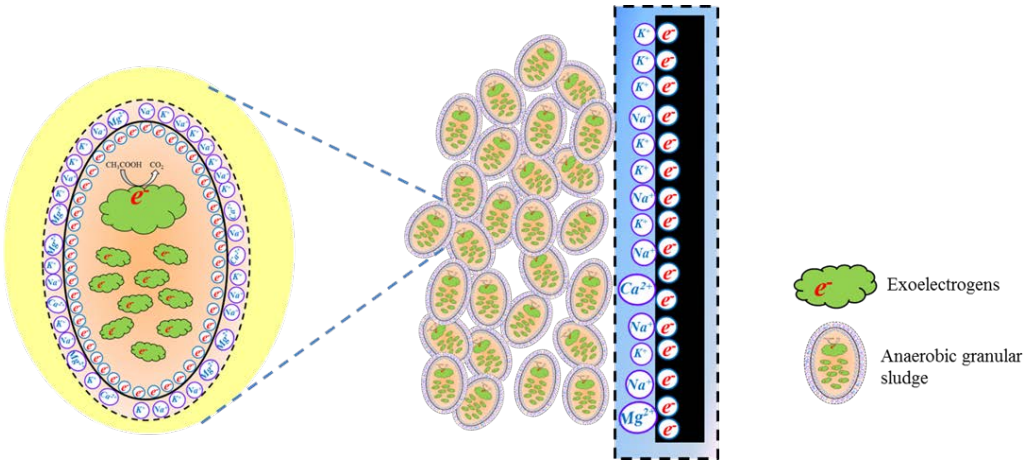

Figure 20. Schematic diagram of the bio-capacitor mechanism

From the results, we could conclude the first two factors were the dominant contributor to the capacitance of the new AGS-based biocapacitor.

To conclude this work, AGS-based biocapacitor was demonstrated to be employed as energy storage device, and optimal anodic potential was +0.2 V and time period was 5 min charging – 10 min discharging. Electrochemical characterization of single granule proved the conductive spots in the connection points. Finally, a new mechanism was proposed to explain how device worked. Taken together, the biggest advantage of the new biocapacitor is to combine the double layer effect with exoelectrogens for electron storage.

However, it has to be mentioned that if we want to precisely differentiate the contribution of each effect for the fundamental research work, it would be better to simplify the current system and utilize modelling to provide supplementary proof.

5 Conclusions

This thesis mainly focused on the extended applications of MET in ammonia biosensor, wastewater treatment and biocapacitor, and fundamentally investigated the electron transfer in anodic biofilm to maintain an efficient MET process. Firstly, an EC-based system and a MEC-based system coupled with nitrification stage were developed separately for online ammonia monitoring. The operational parameters were optimized to reach a stable, reliable and robust ammonia biosensor. The formation of thick, dense and electroactive biofilm was deciphered as a determining factor for an efficient MET process. Thus, more fundamental research exploration of electron transfer pathway in the biofilm was conducted. An effective carbon removal along with electricity generation was realized in an AGS-based MFC. Subsequently, the capacity of electron storage of AGS was evaluated and optimized with multiple parameters. The mechanism of electron storage was stated based on the electrochemical characterization and Raman spectra. Specifically, the main findings are summarized:

- An integrated EC-nitrification system was successfully set up and demonstrated as an effective approach to monitoring ammonia in AD digesters.
- In the nitrification stage, good conversion was achieved from ammonia to nitrate. In EC stage, a linear relationship was observed for current signals and nitrate (ammonia) levels.
- The sensor performance was independent of the external voltage and pH. And the post-treatment method was proved to be effective to eliminate the disturbance of other potential electron acceptors (Fe^{3+} and NO_3^-).
- The results obtained by the sensor for real AD effluents were comparable to the results tested by the testing kits.
- The MEC-nitrification system was successfully employed as ammonia biosensor, and fully utilized the energy in the wastewater to offset the energy consumption.
- The linear relationship between current response and ammonia levels was observed regardless of the external voltage and wastewater pH.
- The accuracy of this biosensor was proved by the real waste streams.
- A dense, thick and electroactive biofilm was deciphered as the determining factor for an efficient MET process.

- AGS was selected as the optimal biocatalyst owing to the innate massive microbes and large surface area.
- Controlling anodic potential at 20 mV successfully shifted the methanogenic AGS to exoelectrogenic AGS.
- 100% carbon removal was obtained when controlling anodic potential at +20 mV, and this strategy proved to be effective to have a persistent effect on the long-term running regarding to the exoelectrogens enrichment and methanogen inhibition.
- The AGS-based MFC was demonstrated to be robust in acid pH conditions.
- The relative abundance of *Desulfurmonadales* spp. increased significantly after the positive anodic potential control, whereas *Methanosaeta concilii* and *Mesotoga infera* decreased as expected.
- A good electrochemically activity of AGS in a new developed biocapacitor system was obtained and indicated by CA, CV and polarization test. The formal potential deciphered from first derivative curve of CV profile was in good accordance with *Geobacter*.
- A good electron storage behavior of AGS was demonstrated via cycle test, and +0.2 V and 5 min charging – 10 min discharging were selected as the optimal operational parameters. Furthermore, by removing AGS, it was suggested the crucial role of AGS in the capacitance contribution.
- Single granule growing on flat graphite and growing within wrapped gold wire proved a conductive electron flow in the connection spot between granule and conductive material. CV profiles and Raman spectra supported the deduction.
- Microbial community analysis verified the appearance of *Geobacter*.
- A mechanism of electron storage in AGS was proposed, mainly including the double layer effect of anode and granular structure, and the redox cofactors (cytochromes) in exoelectrogens.

6 Future perspectives

In this thesis, the MET-based systems were developed for effective wastewater treatment, electron storage device and ammonia monitoring. Operational parameters were investigated and optimized. The working principles contained in each system were discussed as well. However, to get a deeper understanding of the fundamental knowledge of electron storage capacity of AGS, and facilitate the commercialization of ammonia biosensor, more works are still needed.

I About the ammonia biosensor, simplifying the reactor configuration should be done, for example, coupling nitrification into the EC/MEC cathode. The modification of electrode material should be made to improve the performance and reduce the operational cost. Furthermore, long-term monitoring should be employed and real in-situ test should be applied. Downstream optimization should be considered to make it portable.

II In the AGS-based MFC, nutrient (N or P) removal and recovery should be investigated during wastewater treatment. The anodic potential should be optimized to decipher the least energy necessary for AGS transition. Operational cost should be calculated including the energy input of the potentiostat, and compared to the conventional aerobic method. Long-term (over 1 year) operation should be considered to investigate the wastewater treatment performance after the optimal strategy.

III In the AGS-based biocapacitor, more research work about the spatial microbial community should be done. The techniques include fluorescence in situ hybridization for the slice cut via crymicrotome, RNA analysis in terms of the slices. The main goal of this analysis is to target on the helpful information of the microbial distribution in the interior and periphery of single AGS. It would help to decipher the active role of microbes and connection with conductive material.

IV In terms of the biocapacitor evaluation, Ragone plot reporting the energy and power density output should be made to understand the difference/advantages/optimal application field for this biocapacitor compared to the batteries or EC capacitor.

V More work should be done to investigate the electron storage of single AGS, which may help to analyze the resistance of electron transfer within granules. Effective methods should be proposed to diminish the obstruction.

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In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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